Predicting Viral Clearance through CHO-RVLP and MVM-Mock Viral Particles: Characterization, Analysis and Utility in Downstream Process Development Activities

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Introduction

Viruses can arise during the manufacture of biopharmaceuticals through contamination of exogenous viruses or endogenous expression of viral sequences. Regulatory agencies therefore require "viral clearance" validation studies for each biopharmaceutical prior to approval. These studies demonstrate the manufacturing process' ability at removing or inactivating virus and are conducted by challenging scaled-down manufacturing steps with a "spike" of live virus. These studies are conducted in BSL-2 facilities and are costly. Due to these hurdles, process knowledge pertaining to viral clearance is limited during development and characterization. The use of an accurate, economical and quantifiable non-infectious viral surrogate would enable downstream purification scientists to study viral clearance throughout process development.

Stock Solutions of non-infectious CHO-Retrovirus Like Particles (RVLP) and Minute Virus of Mice—Mock Virus Particles (MVM-MVP) have been generated and used as economical spiking surrogates for Xenotropic Murine Leukemia Virus (XMuLV) and Minute Virus of Mice (MVM), respectively. Discussed here are results from physicochemical characterization analysis, methods of quantification, and data from RVLP and MVM-MVP clearance studies. These studies, spanning multiple modes of chromatography and filtration, demonstrate the value of utilizing these non-infectious agents for process development and characterization.

Particle Production and Characterization

Non-infectious RVLP were produced during CHO cell cultivation and purified via multiple modes of chromatography before being concentrated to a final stock solution of 1 x 10^{10} particles/mL. MVM-MVP were assembled after recombinantly expressing MVM's major structural protein (VP2) in a baculovirus/Sf9 system. Particles were then purified via affinity and IEX chromatography, resulting in a final stock solution of 1 x 10^{12} particles/mL.. Figure 1 shows Transmission Electron Microscopy (TEM) images of each particle.

RVLP diameter and net-surface charge were assessed via Dynamic Light Scattering (DLS) and Zeta Potential measurement. For comparison, XMuLV (produced by Texcell, N.A.) was analyzed as well. The DLS results (Figure 2) demonstrate that the RVLP, contained in the stock solution, are monodisperse and exhibit an average diameter of 193 nm while XMuLV, in spiking solution, are also monodsisperse but exhibit a slightly larger diameter (Note: Both measured diameters are higher than those reported for XMuLV by TEM). Prelliminary Zeta Potential results (Table 1) indicate that surface charges, measured at pH 8.6, appear to be similar.

Through a collaboration with the FDA, the physicochemical properties of MVM-MVP were studied and compared to live MVM and PP7 bacteriophage (Johnson, 2017). For physical comparisons, TEM and Multi-angle Light Scattering (MALS) analyses were performed. For surface charge and hydrophobicity, each particle was analyzed via Chromatofocusing and Solute Surface Hydrophobicity techniques. Table 2 summarizes the results from these techniques.

Table 2. MVM-MVP Physicochemical Comparison Summary

* Reference value from Lute et. al. PDA J Pharm Sci Technol (2008)
** Relative hydrophobic affinity to Phenyl (1.0 = insulin)

Analysis	Live MVM	MVM-MVP	PP7 Bacteriophage
Hydrodynamic Radii (MALS)	18.4 ± 0.2 nm	17.2 ± 0.1 nm	16.9 ± 0.4 nm
Diameter (TEM)	24.6 ± 3.6 nm	25.6 ± 3.0 nm	31.6 ± 1.6* nm
Surface Charge (pI)	5.99	5.81	4.74
Hydrophobicity**	0.28	0.35	0.61

Figure 1. TEM Images of RVLP and MVM-MVP

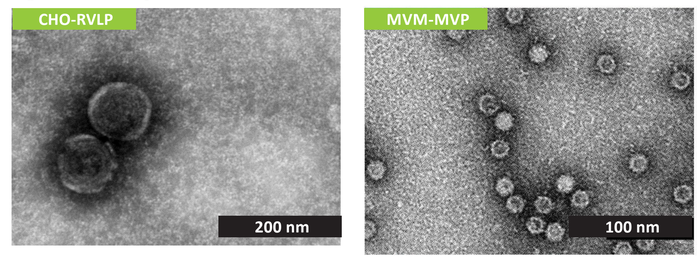
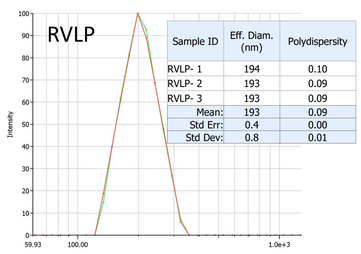
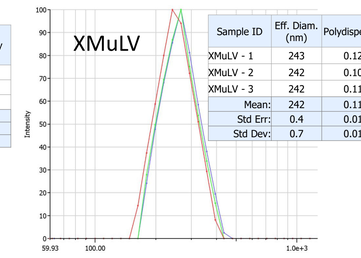


Figure 2 & Table 1. DLS and Zeta Potential results for RVLP and XMLV .



Sample ID	Eff. Diam. (nm)	Polydispersity
RVLP- 1	194	0.10
RVLP- 2	193	0.09
RVLP- 3	193	0.09
Mean:	193	0.09
Std Err:	0.4	0.00
Std Dev:	0.8	0.01



Sample ID	Eff. Diam. (nm)	Polydispersity
XMuLV - 1	243	0.12
XMuLV - 2	242	0.10
XMuLV - 3	242	0.11
Mean:	242	0.11
Std Err:	0.4	0.01
Std Dev:	0.7	0.01

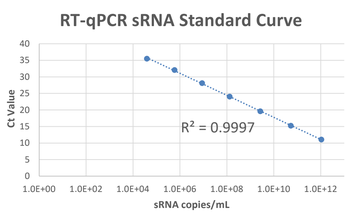
pH	Particle	Zeta Potential (mV)
8.6	RVLP	-19.91
	XMuLV	-16.25

Quantification

CHO-RVLP

To analyze the concentration of noninfectious RVLP in samples, an RT-qPCR method modified from De Wit, 2000 is employed. In short, samples are added to a 96 deepwell plate and are treated with DNAse to degrade CHO-endogenous RVLP DNA sequences. Next samples are diluted in the 96 deepwell plate with an Assay Diluent and treated with Proteinase K for 30 minutes at 60°C. RNA is then extracted and precipitated with a set of proprietary buffers (Cygnus Technologies, LLC). The plate is then stored at -20 °C for 30 minutes and RNA is then pelleted via centrifugation at 3,000 x g for 20 minutes at 4 °C. After removing the supernatant, the pellets are washed, resuspended and re-pelleted via centrifugation twice. After final pelleting, the RNA is resuspended in a proprietary buffer. 2 µL of sample is transferred from each well of the 96 deepwell plate to a qPCR plate containing TaqMan primers/probe directed against the *pol* region of the CHO-RVLP genome. To determine the quantity of particles in unknown samples, threshold cycle (Ct) values are interpolated into a standard curve generated by including a 10-fold dilution series of a known sRNA standard (Figure 3). From those concentration values, RVLP LRVs for each experiment are calculated.

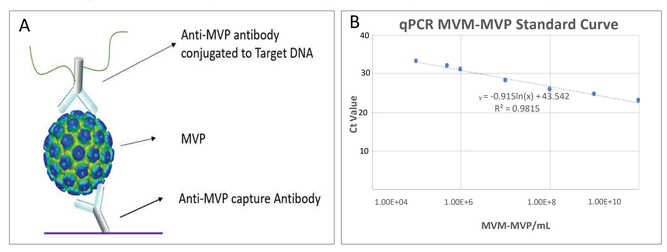
Figure 3. Example sRNA standard curve for RVLP RT-qPCR assay



MVM-MVP

To analyze the concentration of noninfectious MVM-MVP in samples, an Immuno-qPCR assay is performed (Cetlin, 2018, Figure 4a). In brief, samples are added to microwells coated with an anti–MVM-MVP capture mAb. After incubation and washing, a DNA-conjugated anti–MVM-MVP detector mAb is added. Following another incubation and washing step, a dissociation buffer is added to each well for five minutes. Then 5 µL of sample is transferred from each well to a qPCR plate containing TaqMan primers/probe directed against the conjugated DNA. To determine the quantity of particles in unknown samples, threshold cycle (Ct) values are interpolated into a standard curve generated by including a 10-fold dilution series of a known MVM-MVP standard (Figure 4b). From those concentration values, MVM-MVP LRVs for each experiment are calculated.

Figure 4. Immuno-qPCR schematic (a) and example standard curve (b)



Proof of Concept Studies

CHO-RVLP

Protein A Studies

RVLP Stock Solution was spiked into representative mAb Protein A Load (1% v/v), filtered through a 0.45µm load filter and processed through a Protein A column operated under centerpoint conditions. Samples from each phase were taken and analyzed via RT-qPCR after RNA extraction (as detailed above). The LRV results were compared to historical XMuLV data generated through qPCR analysis (Table 3).

Table 3: Protein A RVLP vs. XMuLV (qPCR) LRV Results

Sample	Total RVLP	% RVLP	RVLP LRV	XMuLV LRV
ProA Filtered Spiked Load	2.51E+09	40.9%	NA	NA
ProA Load Flowthrough	1.79E+09	71.2%	0.15	0.16
ProA Wash 1	2.42E+08	9.6%	1.02	1.13
ProA Wash 2	4.53E+06	0.2%	2.74	3.26
ProA Elution	5.04E+07	2.0%	1.70	2.25
ProA Strip	8.19E+06	0.3%	2.49	2.88

AEX Studies

RVLP Stock Solution was spiked into representative mAb in-process material (2% v/v), filtered through a 0.45µm load filter and processed through an AEX column operated in Flowthrough (FT) mode under centerpoint and high conductivity (31 mS/cm) conditions. RVLP Spiked material was also challenged through an AEX column operated in Bind/Elute (B/E) mode under centerpoint conditions. Samples from each phase were taken and analyzed via RT-qPCR after RNA extraction (as detailed above). The LRV results demonstrate complete removal of RVLP by AEX operated in either FT or B/E mode at centerpoint conditions (as expected) but demonstrate RVLP breakthrough during high conductivity operation in FT mode (as expected) (Table 4).

Table 4: AEX RVLP LRV Results

Mode/Operation	Sample	Total RVLP	% RVLP	RVLP LRV
FT / Centerpoint	Filtered Load	5.36E+10	NA	NA
	Flowthrough/Pool	≤ 2.37E+06	≤ 0.01%	≥ 4.35
	Strip	7.76E+09	14.47%	0.84
FT / High Conductivity	Filtered Load	8.16E+10	NA	NA
	Flowthrough/Pool	3.99E+09	4.89%	1.31
	Strip	2.59E+10	31.70%	0.50
B/E / Centerpoint	Filtered Load	5.44E+09	NA	NA
	Flowthrough	≤ 3.74E+05	≤ 0.01%	≥ 4.16
	Wash	≤ 5.31E+05	≤ 0.01%	≥ 4.01
	Elution	≤ 6.00E+05	≤ 38.61%	≥ 3.96
	Strip	2.10E+09	≤ 0.01%	0.41

MVM-MVP

IEX HTS Studies (in collaboration with NIH NIAID-VRC)

The Purpose of this study was to utilize MVM-MVP to screen the performance of AEX and CEX resins from various vendors across a range of pH/Cond conditions while utilizing high throughput automation (Tecan). Robocolumns were first equilibrated with buffer containing 10 mM NaCl (pH 6.5, 7.5, 8.5 for AEX; pH 5.5, 6.5 for CEX). Then, pH-adjusted load (vaccine) was spiked to 1E11 MVM-MVP/mL and added to each column. The plate was mixed and centrifuged while unbound flow through was collected. A series of buffers with increasing NaCl concentrations were added to the columns. After each addition, the plate was mixed, centrifuged and sample were collected. All samples were analyzed for MVM-MVP and LRV's were determined (Figure 5).

Figure 5. LRV results from each fraction collected during anion exchange HTS studies

Resin/pH	Load FT	50mM NaCl	100mM NaCl	150mM NaCl	200mM NaCl	250mM NaCl	300mM NaCl	350mM NaCl	500mM NaCl	1M NaCl
Toyopearl DEAE-650M pH 6.5	4.80	4.66	1.40	0.64	0.63	0.63	0.63	0.63	0.63	0.63
Toyopearl DEAE-650M pH 7.5	4.80	4.66	4.55	1.16	0.73	0.72	0.72	0.72	0.72	0.72
Toyopearl DEAE-650M pH 8.5	4.80	4.66	4.55	2.01	0.77	0.74	0.73	0.73	0.73	0.73
Poros 50D pH 6.5	4.80	4.66	4.55	0.75	0.69	0.67	0.67	0.66	0.66	0.66
Poros 50D pH 7.5	4.80	4.66	4.55	3.18	0.94	0.86	0.84	0.83	0.82	0.82
Poros 50D pH 8.5	4.80	4.66	4.55	1.19	0.94	0.87	0.87	0.86	0.86	0.86
Poros 50PI pH 6.5	4.80	4.66	4.55	4.47	4.40	4.34	4.28	1.27	0.98	0.93
Poros 50PI pH 7.5	4.80	4.66	4.55	4.47	4.40	4.34	4.28	3.95	0.73	0.71
Poros 50PI pH 8.5	4.80	4.66	4.55	4.47	4.03	3.81	3.67	1.95	0.85	0.83
Poros 50HQ pH 6.5	4.80	4.66	4.55	4.06	0.66	0.65	0.65	0.65	0.65	0.65
Poros 50HQ pH 7.5	4.80	4.12	4.09	4.06	2.08	0.95	0.94	0.93	0.93	0.93
Poros 50HQ pH 8.5	4.80	4.66	4.55	4.47	4.40	0.97	0.95	0.94	0.94	0.94
Cytiva Q SFF pH 6.5	4.80	4.66	4.55	4.47	2.45	1.08	0.91	0.90	0.89	0.89
Cytiva Q SFF pH 7.5	4.80	4.66	4.55	4.47	4.40	2.30	1.00	0.76	0.74	0.73
Cytiva Q SFF pH 8.5	4.80	4.66	4.55	4.06	4.03	3.81	1.37	0.93	0.84	0.83
EMD Fractogel TMAE HiCAP pH 6.5	4.80	4.66	4.55	0.61	0.54	0.53	0.53	0.53	0.53	0.53
EMD Fractogel TMAE HiCAP pH 7.5	4.80	4.66	4.55	4.47	0.57	0.49	0.48	0.48	0.48	0.48
EMD Fractogel TMAE HiCAP pH 8.5	4.80	4.66	4.55	4.47	1.14	0.81	0.80	0.80	0.80	0.79
EMD Fractogel DEAE (M) pH 6.5	4.80	4.66	4.55	1.87	0.79	0.77	0.77	0.77	0.77	0.73
EMD Fractogel DEAE (M) pH 7.5	4.80	4.66	4.55	4.47	0.98	0.58	0.57	0.56	0.56	0.56
EMD Fractogel DEAE (M) pH 8.5	4.80	4.66	4.55	1.09	0.79	0.78	0.77	0.77	0.77	0.77
Toyopearl NH2-750F pH 6.5	4.80	4.66	4.55	4.47	4.40	4.00	3.97	3.95	3.93	1.00
Toyopearl NH2-750F pH 7.5	4.80	4.66	4.55	4.47	4.40	4.34	4.28	4.28	4.23	1.23
Toyopearl NH2-750F pH 8.5	3.80	3.78	3.76	3.75	3.73	3.61	3.60	3.59	3.58	1.08

Full data published in Gulla, K. C., et. al. 2021

AAVX Study (in collaboration with REGENXBIO, Thermo Fisher Scientific and Texcell)

The Purpose of this study was to understand the viral clearance potential of Thermo Fisher Scientific's AAVX resin in a representative downstream AAV process and to determine the predictive ability of utilizing MVM-MVP's for AAV viral clearance. AAVX resin was packed into columns (5 mL CV) and qualified. "Centerpoint" and "Worst Case" runs were conducted according to REGENXBIO process parameters. For each run, in-process AAV material, provided by REGENXBIO, was spiked with either MVM-MVP (to a target 10.0 $\log_{10}$ MVP/mL), or live MVM (Texcell). For centerpoint runs, 150 mL's of spiked material was loaded, for worst case runs, 200 mL's was loaded. Samples were collected throughout each process phase and stored at -80 °C prior to Immuno-qPCR or $TCID_{50}$ analysis. From these results, LRV's were determined and compared (Tables 6, 7 and Figure 6).

Table 6. MVM-MVP Clearance Results

		Total $\log_{10}$ MVP	% of MVP	LRV
Run 1 Centerpoint	Load	12.3		
	FT	12.0	52.6%	
	Wash 1	10.0	0.5%	
	Benzonase Wash	11.3	10.4%	
	Wash 2	8.7	0.1%	
	Elution	7.4	0.0%	4.91
	CIP	6.9	0.0%	
Run 2 Centerpoint	Load	12.2		
	FT	12.0	67.0%	
	Wash 1	9.7	0.3%	
	Benzonase Wash	11.2	10.4%	
	Wash 2	8.7	0.0%	
	Elution	7.0	0.0%	5.16
	CIP	6.7	0.0%	
Higher Load Ratio + Residence Time	Load	11.9		
	FT	11.8	79.1%	
	Wash 1	9.9	1.1%	
	Benzonase Wash	11.0	14.3%	
	Wash 2	9.0	0.1%	
	Elution	7.8	0.0%	4.07
	CIP	6.8	0.0%	

Figure 6: AAVX MVM vs. MVP Comparison

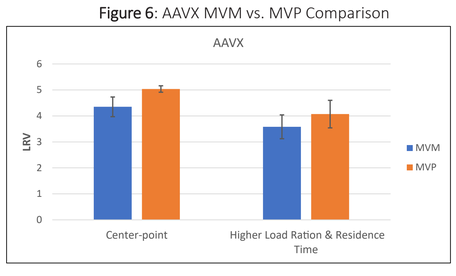


Table 7. MVM Clearance Results

		Total $\log_{10}$ MVM	% of MVM	LRV
Center-point	Load	8.18		
	FT	7.92	54.9%	
	Wash 1	6.10	0.8%	
	Benzonase Wash	5.37	0.2%	
	Wash 2	4.68	0.0%	
	Elution	3.75	0.0%	4.35 ± 0.38
	CIP	4.98	0.1%	
Higher Load Ratio + Residence Time	Load	7.89		
	FT	7.63	54.8%	
	Elution	4.31	0.0%	3.58 ± 0.46

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- **REGENXBIO:** Mikhail Goldfarb, Michael Winkler, Claire Zhang, Michael Boychyn

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