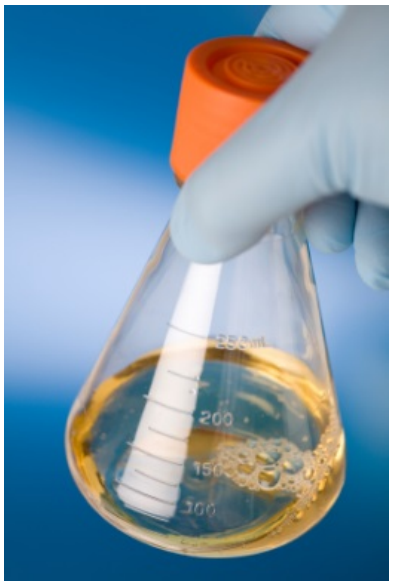
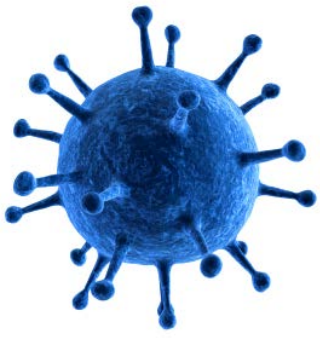




Viroprep®

RAPID VIRUS CLEANUP SYSTEM

New Product Note

"The ViroPrep system is very simple and easy to use for cleaning up virus particles. The instructions are easy to follow and it takes less than 1 hour for the entire process. The kit efficiently eliminates the background debris particles from the samples to almost undetectable levels by EM, and we observed only small loss of virus particles after using the Viroprep procedure."

Peter Pushko, PhD – President, Medigen, Inc.

virocyt

VIRUS QUANTIFICATION MADE SIMPLE

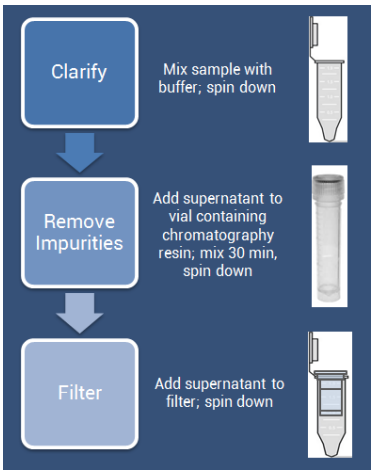
INTRODUCTION

Quantification of viruses has been and continues to be a major chokepoint in a variety of settings. These include viral vaccine research, development and manufacturing, the creation of therapeutic proteins and new vaccine modalities using baculovirus-based expression systems, determining the potency of antivirals, and testing viruses engineered to treat diseases such as cancer. Time-tested approaches, such as plaque titer, tissue culture infective dose, electron microscopy and quantitative PCR all provide valuable insight into the nature of a given virus sample. Unfortunately, each has notable limitations, ranging from cost, time to result, technical complexity, variability, etc. Many of these issues are at least partly the result of directly quantifying crude samples or the delays caused by time-consuming and labor-intensive purification protocols. As a result, there is a pressing need for a rapid, simple-to-use system for removing background components such as protein, nucleic acid, cell debris, leaving a purified virus sample ready for analysis using the previously mentioned methods among others. ViroPrep® Plus is a spin column-based clean-up method developed specifically for this purpose. The system is simple to use, allows parallel processing of up to 12 samples in less than 1 hour, and demonstrates excellent reproducibility and recovery. The system has been tested using primarily egg- and cell-grown influenza. Allantoic fluid is arguably one of the most complex and challenging environments to isolate virus from due to the presence of large amounts of protein, nucleic acid, exosomes (or so-called "blebs") and other components. In this New Product Note, the findings from these experiments are presented, with suggestions for best practices and uses of the technology.

PROCEDURE

ViroPrep Plus is a three-step process:

- 450 µL of crude sample is mixed with 50 µL of 10 x ViroPrep buffer solution to precondition the sample. This solution is then spun down in a benchtop centrifuge at 3000 x g for 5 minutes to remove large debris.
- The supernatant is then removed and added to a slurry containing a multimodal purification resin then allowed to incubate on a shaker for 30 minutes. After incubation, the sample is centrifuged at 1000 x g for 3 minutes to pellet the resin.
- The supernatant is then removed and added to a centrifugal filter assembly and centrifuged at 1000 x g for 3 minutes, eliminating any resin particles and other impurities larger than the pore size from the final sample. The sample is now ready for analysis.



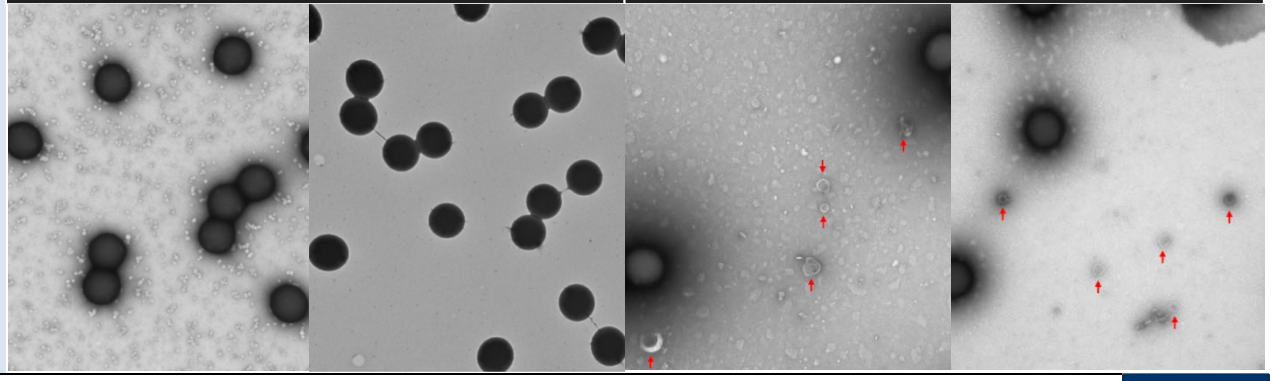
RESULTS

Removing Interfering Particles

Transmission electron microscopy (TEM) was used to characterize samples of infected and mock infected allantoic fluid from several sources (**Figure 1**). Infected samples from both Supplier 1 and Supplier 2 showed particles of 30-50 nm which were reduced significantly (81%-100% removal) after processing with ViroPrep Plus. The observed 30-50 nm particles likely represent egg-derived structures, as mock-infected samples also showed background particles. Interestingly, the quantity of these particles is elevated in influenza-infected samples as compared to uninfected samples.

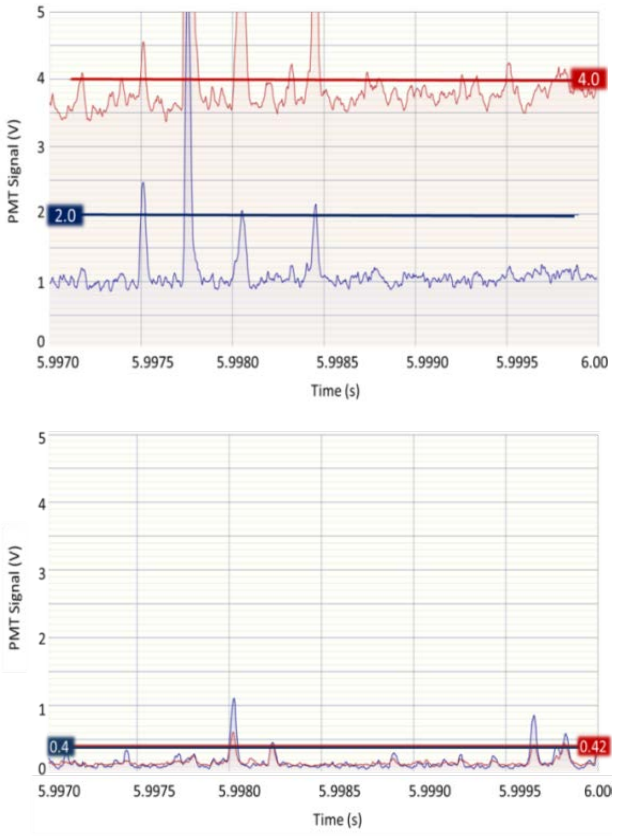
Figure 1. Electron micrographs of allantoic fluid pre- (left) and post- (right) clean up using ViroPrep Plus reveal significant removal of 30-50nm particles. Note: Sizing beads are 500nm in diameter and magnification is 40,000x.

Figure 2. Example of TEM images pre- (left) and post- (right) ViroPrep treatment used to quantify influenza recovery. Red arrows indicate individual viruses. Note: Sizing beads are 500nm in diameter and magnification is 40,000x.



Virus recovery was determined by spiking influenza B/Lee/40 at a known concentration into mock infected allantoic fluid, followed by ViroPrep Plus processing, adding 500 nm standard beads to both pre- and post-ViroPrep samples and visualized by negative staining TEM, as shown in **Figure 2**. Using this approach, an average recovery of 79% was calculated, indicating good retention of the virus of interest, while removing from 81% - 100% of the interfering 30 nm - 50 nm structures.

Figure 3. Representative Virus Counter 2100 screenshots for crude A/California/7/2009 for both pre-ViroPrep (upper panel) and post-ViroPrep (lower panel), highlighting the significant baseline reductions achieved with the ViroPrep method. Data in both examples were collected with no additional dilution applied to the crude starting material.



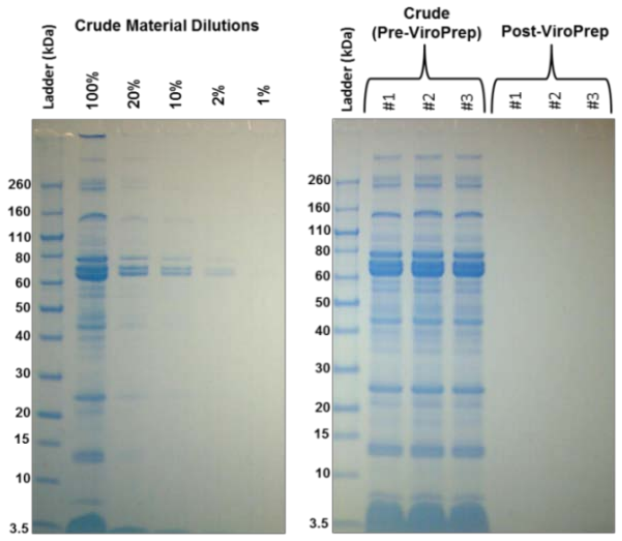
Reducing Background Protein and Nucleic Acid

Next, the Virus Counter® 2100 instrument was utilized to measure the average nucleic acid and protein channel baseline voltages of influenza in allantoic fluid both before and after cleanup with the ViroPrep method. SDS-PAGE was also performed to corroborate these results.

To obtain pre-cleanup baseline measurements, crude influenza-containing allantoic fluid obtained from several sources was stained with Combo Dye prior to cleanup and subsequently analyzed on the Virus Counter. At least 3 replicates of each starting material were analyzed. Due to the complexity and viscosity of the sample matrix, a dilution of the starting sample was necessary prior to staining in order to obtain proper fluidic behavior of the sample. Therefore, the unpurified material could not be analyzed undiluted on the Virus Counter 2100. After ViroPrep, the purified influenza-containing samples were stained with Combo Dye and analyzed on the Virus Counter 2100 to examine the reduction in average baselines on both the protein and nucleic acid channels.

To complement the protein baseline data from the Virus Counter, SDS-PAGE was carried out pre- and post-ViroPrep to assess the reduction in protein content. Several dilutions of the starting material were also analyzed to allow a semi-quantitative comparison to the post-ViroPrep material.

Figure 4. SDS-PAGE results of crude egg-grown A/Victoria both before and after cleanup. Gel on left shows several dilutions of starting material, where percentages shown at top of each lane indicate the amount of crude starting material added to each lane. Gel on right shows thee replicate samples both before and after cleanup.



The pre-ViroPrep Virus Counter and SDS-PAGE data shown in **Figures 3** and **4** both indicate the presence of significant amounts of protein impurities. Post-ViroPrep, impressive baseline reductions shown in **Figure 3**, as measured by final average Virus Counter baselines of less than ~1 V on each channel, were achieved for both the nucleic acid and protein channels for a variety of different crude starting materials (**Table 1**). By examining a serial dilution of the starting material (left-hand side of **Figure 4**), it can be seen qualitatively that the protein is at the limit of detection of the SDS-PAGE gel somewhere between the 50x and 100x dilutions (2% and 1% starting material, respectively), indicating ViroPrep achieved at least a 98% cleanup of the original protein in the sample.

Table 1. Analysis of Virus Counter baseline voltages for crude allantoic samples before and after ViroPrep Plus. Notes: 1. All errors associated with the baseline measurements shown are ± 1 standard deviation for the number of replicate measurements indicated (n=replicate measurements, including both multiple aliquots of same sample as well as replicate measurements on each sample, if performed); 2. Dilution factor required to successfully analyze crude starting material on Virus Counter 2100.

Virus strain	Source	Nucleic Acid Baseline							Protein Baseline						
		Pre-cleanup				Post-cleanup			Pre-cleanup				Post-cleanup		
		dilution[2]	avg. V	std dev (V)[1]	n	avg. V	std dev (V)	n	dilution[2]	avg. V	std dev (V)	n	avg. V	std dev (V)	n
A/H1N1/CA/07/2009 2013-2014 vaccine strain	ViraPur	4x	0.14	0.04	9	0.10	0.01	15	4x	0.19	0.05	9	0.14	0.00	15
A/H3N2/Texas/50/2012 2013-2014 vaccine strain	ViraPur	4x	0.51	0.19	6	0.35	0.01	11	4x	1.41	0.59	6	0.30	0.04	11
B/Wisconsin/1/2010 2012-2013 vaccine strain	ViraPur	4x	0.20	0.00	6	0.16	0.02	17	4x	0.29	0.04	6	0.16	0.01	17
B/Malaysia/2506/2004 2007-2008 vaccine strain	BEI	4x	0.49	---	1	0.52	0.01	9	4x	1.37	---	1	0.67	0.05	9
A/H3N2/Victoria/3/1975 older vaccine strain	ViraSource	16x	1.03	0.34	6	0.59	0.09	6	16x	2.84	0.73	6	1.14	0.15	6

Consistency of Virus Recovery

The relationship between input virus concentration and percent virus recovery through the ViroPrep method was investigated using the recommended negative control matrix outlined below (2x-diluted mock-infected allantoic fluid). Crude egg-grown influenza A/California/4/2009 was spiked in at various concentrations (from 2.0 x 10^9 to 2.5 x 10^7 vp/mL) into the negative control matrix of mock-infected allantoic fluid diluted two-fold in purified 0.02 µm-filtered 18 MΩ water. The negative control matrix with no virus added was also analyzed. A first experiment was conducted in which a single operator processed and analyzed three replicate dilution series (total of 27 samples). In a second experiment, two unique operators each processed and analyzed a single dilution series (9 samples per operator). All starting samples were also analyzed via quantitative reverse transcription polymerase chain reaction (qRT-PCR) and hemagglutination assay (HA). The ViroPrep method was then performed on each sample, and qRT-PCR, HA assay and Virus Counter measurements were obtained post-ViroPrep. Percent virus recoveries were determined both from qRT-PCR and HA measurements. The qRT-PCR and HA results shown in **Table 2** indicate that the percent recovery is independent of virus concentration over the concentration range examined. For the single operator/triplicate samples experiment, average recovery of 28 ± 5% by qRT-PCR analysis and 50% by HA analysis were obtained.

Table 2. Standard addition experiments investigating percent recovery as a function of concentration. The top table is an experiment in which three replicates of a dilution series were processed by a single operator. The bottom table details a follow-up study in which two operators each processed one set of serial dilutions.

Dilution Factor	qRT-PCR (copies/mL)			Hemagglutination Assay (HAU/100 µL)			Virus Counter (vp/mL)
	Pre-ViroPrep	Post-ViroPrep	%Recovery	Pre-ViroPrep	Post-ViroPrep	%Recovery	Post-ViroPrep
0	(1.8 ± 0.1) x10^9	(5.7 ± 0.6) x10^8	32% ± 4%	512	256	50%	(2.9 ± 0.8) x10^8
2x	(9.7 ± 0.3) x10^8	(3.2 ± 0.4) x10^8	33% ± 4%	256	128	50%	(1.8 ± 0.3) x10^8
4x	(5.9 ± 0.4) x10^8	(1.5 ± 0.2) x10^8	25% ± 4%	128	64	50%	(5.5 ± 0.4) x10^7
8x	(2.7 ± 0.08) x10^8	(7.3 ± 1.4) x10^7	27% ± 5%	64	32	50%	(2.4 ± 0.1) x10^7
16x	(1.6 ± 0.05) x10^8	(4.0 ± 0.7) x10^7	24% ± 4%	32	16	50%	(1.6 ± 0.6) x10^7
32x	(9.1 ± 0.4) x10^7	(2.2 ± 0.5) x10^7	24% ± 6%	8	8	100%	(1.0 ± 0.3) x10^7
64x	(6.0 ± 0.3) x10^7	(1.3 ± 0.4) x10^7	23% ± 6%	None Detected		N/A	(8.0 ± 1) 10^6
128x	(2.6 ± 0.06) x10^7	(9.8 ± 0.9) x10^6	38% ± 3%	None Detected		N/A	---

Dilution Factor	qRT-PCR (copies/mL)			Hemagglutination Assay (HAU/100 µL)			Virus Counter (vp/mL)
	Pre-ViroPrep	Post-ViroPrep	%Recovery	Pre-ViroPrep	Post-ViroPrep	%Recovery	Post-ViroPrep
0	(1.8 ± 0.05) x10^9	(7.1 ± 0.7) x10^8	40 ± 4 %	256	128	50%	(3.6 ± 0.2) x10^8
2x	(8.3 ± 0.4) x10^8	(3.8 ± 0.1) x10^8	46 ± 3 %	128	64	50%	(2.2 ± 0.07) x10^8
4x	(4.9 ± 0.4) x10^8	(2.5 ± 0.05) x10^8	52 ± 2%	64	32	50%	(1.1 ± 0.2) x10^7
8x	(3.1 ± 0.08) x10^8	(1.0 ± 0.08) x10^8	33 ± 3%	32	8	25%	(3.8 ± 0.8) x10^7
16x	(1.6 ± 0.07) x10^8	(7.1 ± 0.4) x10^7	46 ± 3%	None Detected	2	---	(1.9 ± 0.5) x10^7
32x	(9.9 ± 0.3) x10^7	(3.6 ± 0.4) x10^7	36 ± 4%	None Detected		---	(1.5 ± 0.2) x10^7
64x	(5.4 ± 0.1) x10^7	(2.1 ± 0.1) x10^7	38 ± 3%	None Detected		N/A	(1.0 ± 0.1) 10^7
128x	(3.5 ± 0.01) x10^7	(1.4 ± 0.05) x10^6	38 ± 1%	None Detected		N/A	(7.1 ± 1) 10^6

Figure 5 shows the Virus Counter results for the two sets of standard addition experiments, indicating linearity between measured concentration and dilution factor. For both datasets, the slope is close to 1.0, providing additional evidence of constant percent recovery as a function of concentration. All results at or below the sample quantification limit (SQL) of 7.5 x 10^6 vp/mL (dotted line in Figure 3) were not included in the trendline.

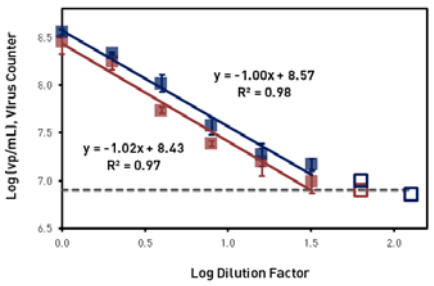


Figure 5. Virus Counter results for standard addition experiments. The series in red corresponds to the top of Table 3 (single operator, triplicates), and the blue series corresponds to the bottom of Table 3 (two operators, single set). Dotted line represents sample quantification limit (SQL) determined.

SUMMARY

The results presented in this New Product Note clearly demonstrate the ability of the ViroPrep Plus system to quickly and easily clean up crude, virus-containing samples. This allows for subsequent quantification using either infectivity or particle counting approaches where the presence of high levels of proteins, nucleic acids, as well as larger structures such as exosomes would otherwise interfere with or prevent these types of analyses. These include but are not limited to plaque titer, tissue culture infectious dose, egg infectious dose, fluorescence focus assay, electron microscopy, quantitative PCR, HPLC and the Virus Counter 2100. By avoiding costly, time-consuming viral purification procedures like ultracentrifugation, the development and production of products using viruses can be significantly streamlined.

For more information about ViroPrep Plus, please email us at info@virocyt.com.

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