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Application Note

Intravacc. Innovating vaccines

Effect of Benzonase® Endonuclease Addition to Purification of Sabin Polio Virus Type 3

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Objective

In vaccine and viral vector production, impurity clearance is an essential purification step and required for drug approval as residual impurities may impact patient safety. Additionally, impurities such as extracellular nucleic acids present in process intermediates can interfere with and reduce the efficiency of various purification steps. A critical step in the purification process is reduction in the size and quantity of extracellular nucleic acids by endonuclease enzymes. Benzonase® endonuclease is a genetically engineered nuclease from *Serratia marcescens*, able to attack and degrade all forms of DNA and RNA. Because of its essential role in vaccine and viral vector processes, it is manufactured under GMP according to ICH Q7, and has a Drug Master File in place with the United States Food and Drug Administration (FDA) which can be cited in regulatory filings. Both the World Health Organization (WHO) and the FDA recommend clearance of DNA to a level of less than 10 ng/dose and fragments less than 200 base pairs in length.

This Application Note summarizes the benefits of incorporating Benzonase® endonuclease in a polio virus type 3 production process. Specifically, we discuss:

- The amount of Benzonase® endonuclease required to degrade Vero cell DNA
- The effect of Benzonase® endonuclease on clarification filter capacity
- The impact of Benzonase® endonuclease on the level of Vero cell DNA following tangential flow filtration
- Improvements to concentration using tangential flow filtration
- The ability of Benzonaze® endonuclease to reduce DNA level to less than 10 ng/dose and fragments less than 200 base pairs in length, in compliance with regulatory guidelines

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Materials and Methods

Culture Conditions

Table 1 summarizes study parameters and **Figure 1** provides the experimental scheme with sample codes. Culture of Vero cells was initiated in T-175 flasks, 875 cm^2 multi-flasks and multi-trays. A 16L bioreactor with a 9L working volume was inoculated with ~0.1x10^6 cells/mL; the bioreactor contained 3g/L Cytodex™ microcarrier beads. Set points during the cell culture were as follows:

- pH: 7.2
- Temperature: 37 °C
- Dissolved oxygen: 50%
- Stirrer: 70-130 rpm

A stirrer controller was included in the dissolved oxygen (DO) cascade; if the maximum O_2 output failed to increase the DO percentage, the stirrer speed was increased. Cultures were sampled once per day on weekdays and cells were counted using a NucleoCounter® cell counter (ChemoMetec) and metabolites measured using a BioProfile® analyzer (Nova Biomedical).

When the Vero cells reached a concentration of ~1.0x10^6 cells/mL (~96 h =TOI time of infection), they were infected with Sabin Polio virus type 3 with a multiplicity of infection (MOI) of 0.001. Virus culture conditions at the time of harvest were as follows:

- pH: 7.4
- Temperature: 32.5 °C
- DO: 25%
- Stirrer: 70-130 rpm

Study parameters and experimental results are provided in **Table 2** and **Figure 1**, respectively. On the day of harvest (3 days/69 hours; see **Table 1**), a sample was taken and inspected for cytopathogenic effect (CPE) under the microscope and for turbidity.

USP Culture Conditions	Results
Viable cell density at start (*10E^6 c/mL)	0.059
Cell growth rate (h)$^{-1}$	0.031
TOI (h)	96.2
Viable cell density at TOI (*10^6 c/mL)	1.08
Cell Doubling (h)	22.2
Sabin Polio Virus Strain #	Type 3
MOI	0.001
Duration virus culture (h)	69
Turbidity (NTU)	55.0

Table 1. Vero cell culture and harvest conditions.

Parameter	Description
Culture medium	VPSFM (Gibco™)
Virus Strain	Sabin virus polio Type 3
Virus Medium	VPSFM (Gibco™)
CPE percentage (%)	90-95
Bioreactor working volume (L)	9
Clarification filters	Millistak+® HC C0HC depth filter in µPOD® (23 cm^2) format
	Lab Scale Pod (270 cm^2) Millistak+® HC C0HC depth filter
	Opticap® XL150 Millipore Express® SHC (140 cm^2)
TFF	Pellicon® XL 50 cassette (100 kDa Biomax® membrane)

Table 2. Study parameters.

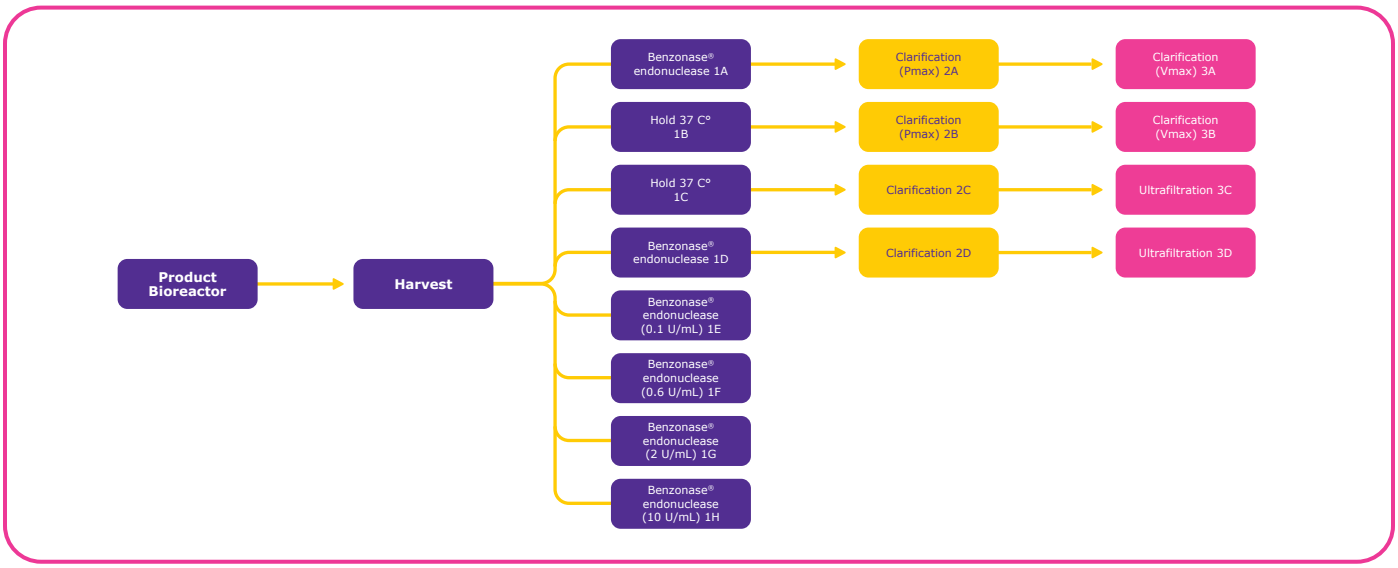

Figure 1. Experimental schemes with samples.

Bioreactor Harvest

Contents of the bioreactor were harvested as outlined in **Figure 2** through a 76 µm sieve to remove the microcarriers. This material was then split into multiple aliquots for treatment with different quantities of Benzonase® endonuclease (**Table 3**). Two samples (1B, 1C) were used as negative controls, with no Benzonase® endonuclease addition. $MgCl_2$ was added to all samples to a final concentration of 2 mM and stirred for 10 minutes.

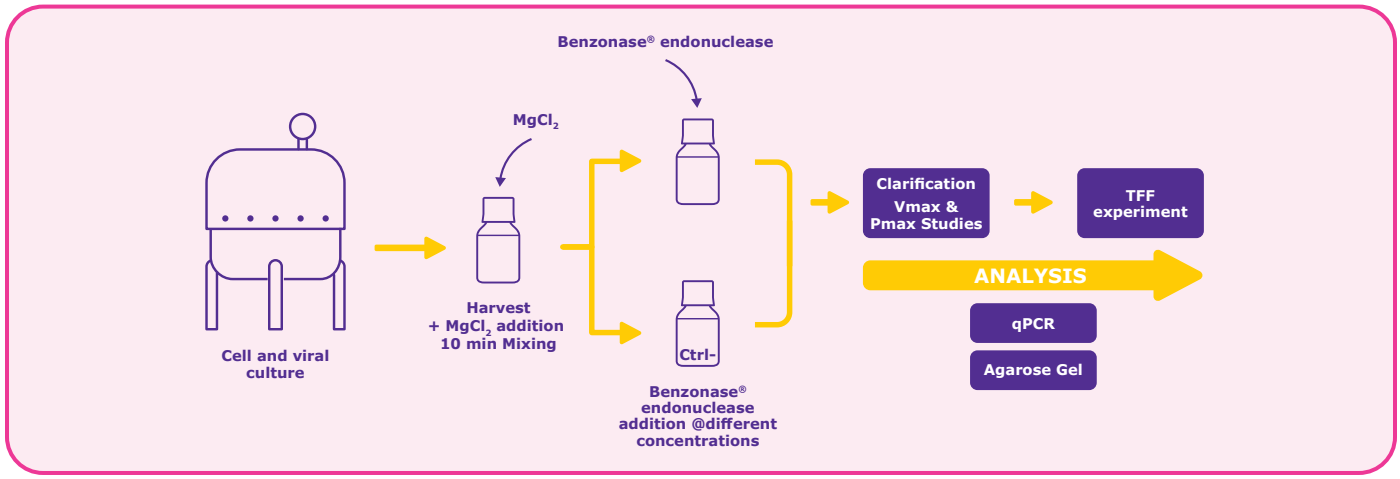

Figure 2. Bioreactor harvest.

P_{max} and V_{max} Trials

Disposable Millistak+® HC C0HC filters in µPod® format (23 cm^2) were used in the P_{max} trials. Prior to use, the filters were preflushed with 400 mL of Milli-Q® water to remove any leachables and then conditioned with 100 mL of cell culture medium. Following a leakage test, the filters were emptied to prevent dilution of the bioreactor harvest. Bulk samples 1A (with Benzonase® endonuclease) and 1B (without) were filtered at a constant flow rate of 11 mL/minute (We recommend 100-150 LMH for filtration flux). The process was stopped when the inlet pressure reached 0.8 bar or all material (1L) had passed through. It should be noted that the P_{max} was measured at a constant flow rate until the pressure reached 0.8 bar whereas the V_{max} was run using a worst-case scenario of 0.9 bar.

The V_{max} trials were performed with OptiScale® 25 capsules (3.5 cm^2) containing sterilizing-grade Millipore Express® SHC membrane. Before use, the filters were preflushed with 70 mL of Milli-Q® water to remove any leachables and then conditioned with 100 mL of cell culture medium. Following a leakage test, the filters were emptied to prevent dilution of the bioreactor harvest. The trials were run with a constant pressure of 0.9 bar for 16 minutes to determine any possible impact of Benzonase® endonuclease on the clarified bulks from the P_{max}™ trials (samples 2A and 2B).

Sample	Units of Benzonase® endonuclease per mL	Bulk harvested Volume (mL)
1A	10	1400
1B	-	1000
1C	-	2100
1D	10	2500
1E	0.1	200
1F	0.6	200
1G	2	200
1H	10	200

Table 3. Volume of Benzonase® endonuclease (stock formula, 346 U/µL) added to harvested volumes.

Clarification

Bulk harvested volumes with (1D) and without (1C) Benzonase® endonuclease were clarified separately by serial depth filtration using a disposable Millistak+® HC C0HC Lab Scale Pod filter (270 cm^2), followed by filtration through an Millipore Express® SHC Opticap® XL150 sterilizing-grade filter. Prior to use, the Millistak+® filters were preflushed with 4L of Milli-Q® water to remove any leachables and then conditioned with 500 mL of cell culture medium. The Millipore Express® SHC filters were preflushed with 80 mL of Milli-Q® water and conditioned with 500 mL of cell culture medium.

Tangential Flow Filtration

Two Pellicon® XL 50 cassettes (100 kDa Biomax® membrane, 50 cm^2) were used to concentrate the clarified bulks with (2D) and without (2C) Benzonase® endonuclease. The process was operated according to the parameters in **Table 4**.

Process Parameters	Process Ranges	2.5L Scale	Set Point
Filter Flushing (Milli-Q® water)			
Volume			
Shear rate	2000 - 5000 sec^{-1}	2000 - 5000 sec^{-1}	4000 sec^{-1}
Flow rate	19-36 mL/min	mL/min	36 mL/min
TMP	≤0.7bar	-	0.6 bar
Delta P	≤1.4 bar	-	1.0 bar
Concentration Factor	100 - 700 x	100 - 700 x	600 - 700 x

Table 4. Process parameters for concentration using TFF.

Results and Discussion

Benzonase® Endonuclease Ranging Study

With use of Benzonaze® endonuclease, we achieved a DNA concentration of less than 10 ng/dose, which is in accordance with regulatory guidelines. Results of the Benzonase® endonuclease ranging study indicated that a concentration of 10 U/mL was effective at cutting the DNA into smaller fragments within one hour (**Figure 3**). Assays for Vero cell residual DNA indicated a 15-fold reduction, from $1.80x10^6$ to approximately $0.12x10^6$ pg/mL, following treatment with Benzonase® endonuclease (samples 1D and 1H, **Figure 2**). Vero cell DNA was also significantly reduced in the bulk 1A which had 4 units of Benzonase® endonuclease added.

A qPCR method was used to evaluate samples following treatment with Benzonaze® endonuclease, but no significant differences were observed in the samples in which lower units of nuclease added (0.1-2 units). These results indicate the required number of Benzonase® endonuclease units necessary to degrade Vero cell DNA was between 4 and 10 U/mL in one hour, but the conditions were not optimized. There was no effect of the Benzonase® endonuclease addition on titer, host cell protein or D-antigen levels of the samples.

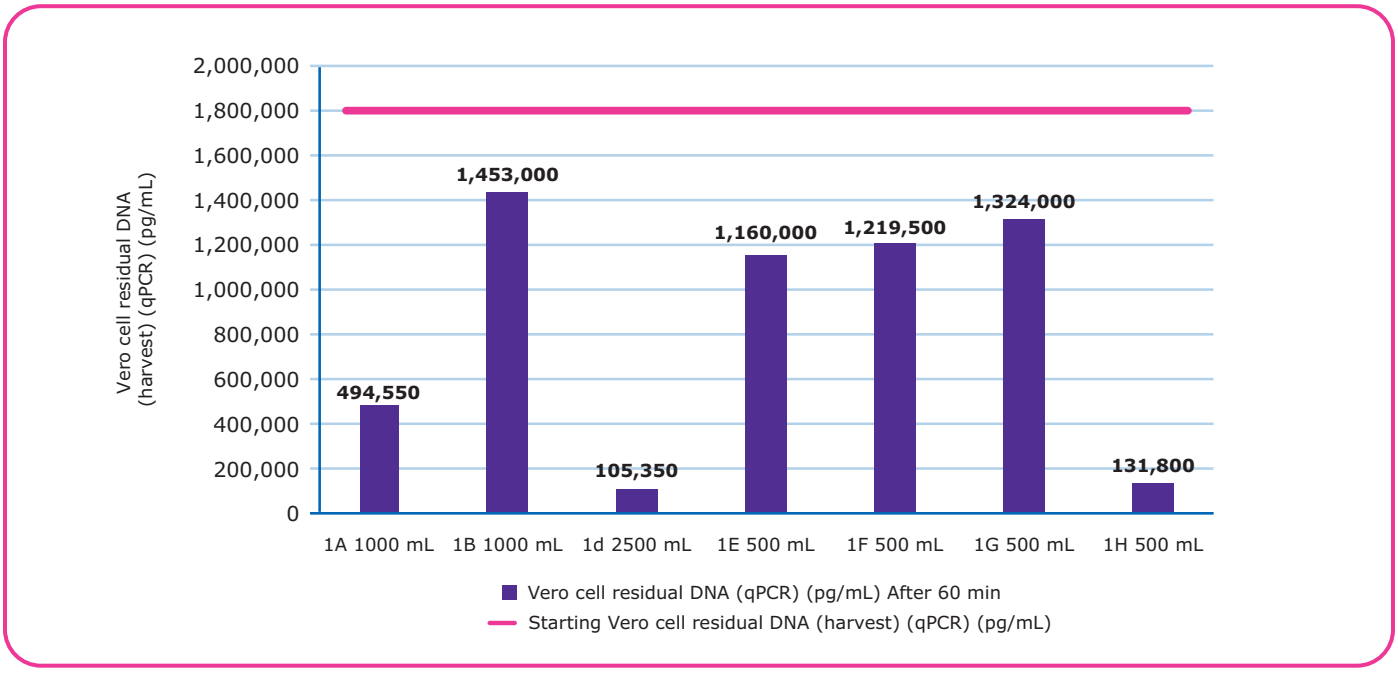

Figure 3. Vero cell residual DNA.

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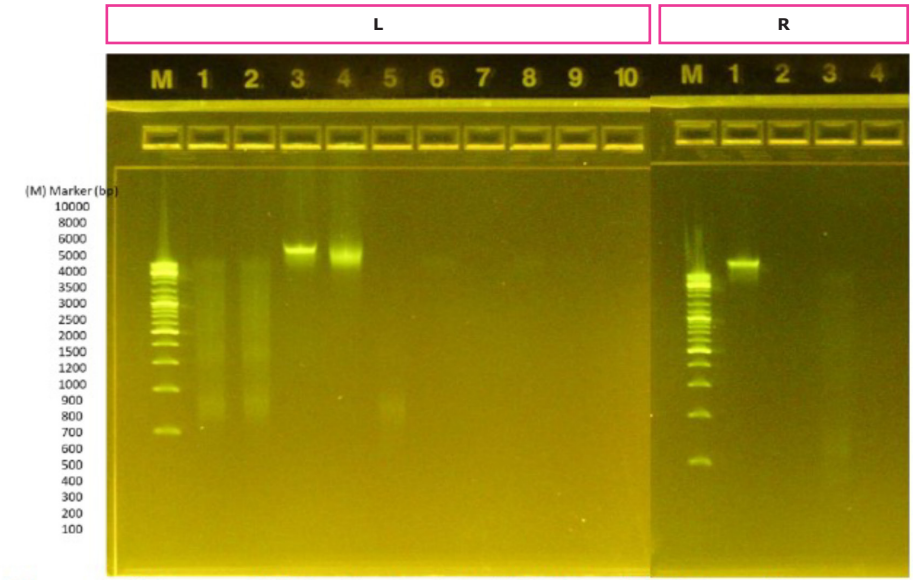
An agarose gel was run with sample DNA to better illustrate the effect of Benzonase® endonuclease on DNA fragmentation (**Figure 4**). For the bulk without addition of Benzonase® endonuclease (lane 3L) and the harvest (lane 1R), a band above 10000 bp can indicate the presence of non-fragmented high molecular weight DNA. Lane 1L and 2L, show the profiles of Benzonase® endonuclease (4 units) digested samples where the spread of fragmented DNA ranging from 500 bp to 4000 bp lengths can be observed.

These results demonstrate the ability of Benzonase® endonuclease to cleave the DNA into smaller fragments which can be removed in subsequent purification steps. For sample 1H (10 units, lane 3R), results showed an efficient DNA digestion in comparison to 1A (4 units), in accordance with what is shown in **Figure 3**. Excessive amounts of Benzonase® endonuclease result in complete DNA cleavage as observed in the positive control (2R).



M	Left or right gel	Marker
1	L	1A-BNZ-2h
2	L	1A-BNZ-30 min
3	L	1B-HOLD-1h
4	L	1E-BNZ-1h
5	L	2A-CLR-PMAX-BNZ
6	L	2B-CLR-PMAX
7	L	3A-CLR-VMAX-BNZ
8	L	3B-CLR-VMAX
9	L	3C-UF
10	L	3D-UF-BNZ
M	R	Marker
1	R	01-harvest
2	R	01-harvest + BNZ 1000U
3	R	1H-BNZ-1h
4	R	NC (Lysis Buffer)

M	Left or Right Gel	Marker	Conditions	Results
1	L	1A-BNZ-1h	Bulk with addition of Benzonase® endonuclease 4 units/mL - 1 hour incubation	A large spread of DNA base pair lengths can be observed for the bulk 1A
2	L	1A-BNZ-30 min	Bulk with addition of Benzonase® endonuclease 4 units/mL - 30 min incubation	
3	L	1B-HOLD-1h	Bulk without addition of Benzonase® endonuclease	Band above 10000bp can be observed
4	L	1E-BZN-1h	Bulk without addition of Benzonase® endonuclease 0.1 unit/mL	Smudge observed at ~8000-10000bp: Benzonase® endonuclease was starting to have an effect. The number of units added appears to be too low or time too short for the DNA to be adequately cut
5	L	2A-CLR-PMAX-BNZ	Post clarification with addition of Benzonase® endonuclease	
1	R	01-Harvest	-	A band above 10000bp can be observed
2	R	01-Harvest+BNZ 1000U	Positive control: 200 units added to 200 µl harvest	No bands appear on the gel due to the excessive amount of Benzonase® Nuclease added
3	R	1H-BNZ-1h	Bulk with addition of Benzonase® endonuclease 10 units/mL - 1 hour incubation	Results equal to 1A (1 L) but fainter bands

Figure 4. Agarose (2%) gels of isolated DNA. Lane 2R was a positive control (1000 U/mL Benzonase® endonuclease) carried out on 200 µl of harvested material stored at -80°C.

Benzonase® Endonuclease Improves Efficiency of Clarification

The flow rate during the Pmax trials was 11 mL/min for samples with and without Benzonase® endonuclease treatment. For the harvested material without Benzonase® endonuclease (2B), the maximum pressure was 0.74 bar (922 mL, 40 mL/cm^2), at which point the filtration using Millistak+® HC C0HC filter was stopped (data not shown).

Breakthrough can be observed in **Figure 5** with a significant drop in pressure observed and increase in NTU. In comparison, the harvested material with Benzonase® endonuclease addition did not show breakthrough and reached a maximum pressure of 0.51 bar (947 mL, <41/cm^2). DNA levels dropped to similar levels both with (59155 pg/mL) and without (55885 pg/mL) Benzonase® endonuclease. Although the levels of DNA in the samples are similar, the DNA in the bulk with the addition of Benzonase® endonuclease has been cut into smaller fragments, compared to no treatment (lanes 5L and 6L, **Figure 3**), and as expected. This yielded shorter fragments of DNA that were more easily eliminated, resulting in faster and more efficient TFF. In addition, optimizing the clarification filtration flux by selecting a value between 4 and 6 mL/min (100 and 150 LMH) could be beneficial to increase the DNA adsorption efficiency.

The flow during V_{max} for the bulk with and without Benzonase® endonuclease was comparable at 67 mL/minute (data not shown). DNA levels dropped to 102 pg/mL (with Benzonase® endonuclease). While this drop was unexpected, the bulks were stored overnight at room temperature after the P_{max} experiment, indicating the Benzonase® endonuclease remained active and continued to cut the DNA into smaller fragments before the V_{max} experiment. The bands were no longer visible on the agarose gel (lane 7L, **Figure 4**) for the bulk with Benzonase® endonuclease whereas a faint band was observed at the top of the gel for the control without Benzonase® endonuclease (lane 8L), showing long stranded DNA. Results indicated that there was no observed effect on the Vmax experiments (data not shown).

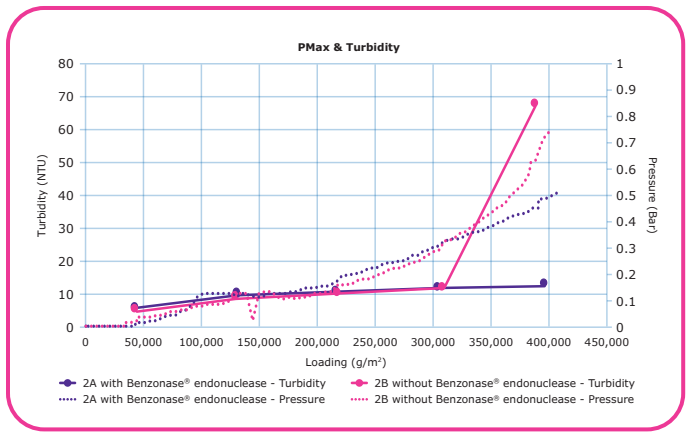


Figure 5. Nephelometric turbidity units (NTU), weight of the filtrate (g) and time during the P_{max} clarification trials with (2A) and without Benzonase® endonuclease (2B).

	3D_UF with Benzonase® endonuclease	3C_UF without Benzonase® endonuclease
Concentration Factor	14.4	11.4
Vero Cell DNA (qPCR)	<30 pg/mL	25810 pg/mL
Titre	9.0 log CCID 50/mL	8.5 log CCID 50/mL

Table 5. Data overview of TFF bulks with (3D) and without (3C) Benzonase® endonuclease.

Benzonase® Endonuclease Improves Efficiency of Tangential Flow Filtration

The filter area of the TFF cassette was small for the volume of material available, therefore the concentration step required a long period of time. Acting on the low flow rate, the transmembrane pressure (TMP) was increased to 0.6 bar based on previous experience with the Polio virus.

A total of 1.2 L of the clarified bulks were concentrated. The TFF proceeded at a faster rate with the bulk containing Benzonase® endonuclease (3D) compared to the bulk without (3C). TMP and pressure profiles of the two bulks are shown in **Figure 6**; the 7 min mobile average of flux divided by TMP and Delta P comparison with and without Benzonase® endonuclease is shown.

During the same time frame, the bulk with Benzonase® endonuclease was concentrated to a factor of 14.4 while the bulk without had a concentration factor of 11.4 (**Table 5**). A large difference was observed in the amount of Vero cell DNA in the bulk without Benzonase® endonuclease (2813.3 ng) compared to the bulk with Benzonase® endonuclease (2.6 ng). This clearly demonstrated the effectiveness of Benzonase® endonuclease to cut the DNA into pieces small enough to pass through the TFF membrane into the permeate. The difference observed in the other assays was due to the bulk with Benzonase® endonuclease being concentrated to a greater extent and as expected, values for titer, total protein, D-antigen and Vero cell host cell proteins were proportionally higher per mL (**Figure 7**).

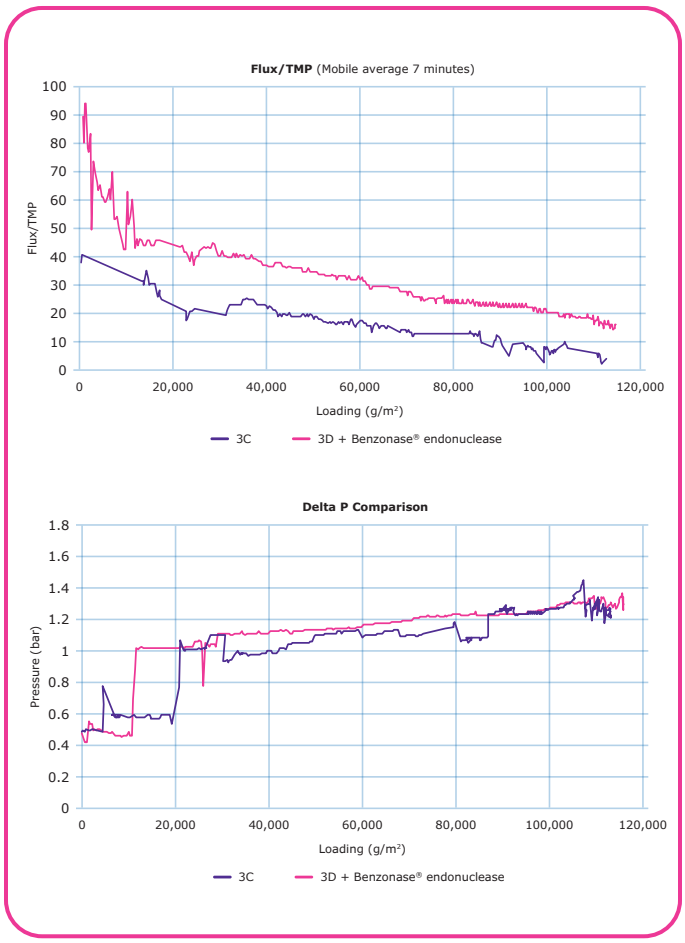


Figure 6. Comparison of 7 min mobile average of flux divided by TMP and Delta P comparison with (3D) and without (3C) Benzonase® endonuclease.

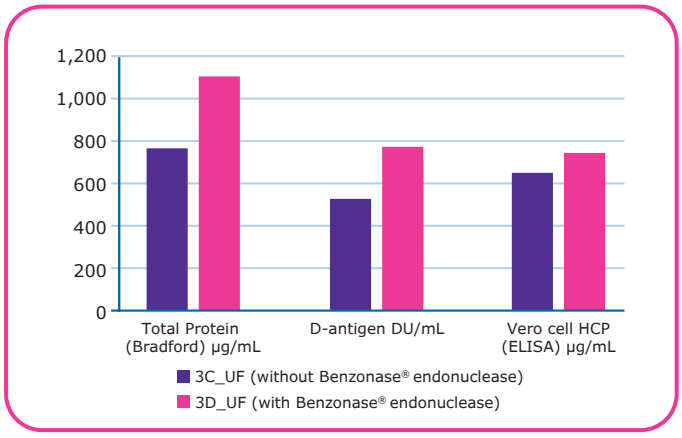


Figure 7. Data overview of TFF bulks with (3D) and without (3C) Benzonase® endonuclease.

Conclusions

Results of this study demonstrate the the ability of Benzonase® endonuclease to reduce levels of Vero host cell DNA in the purification of Sabin Polio virus type 3 vaccine production process (**Figure 8**) A concentration of 4 U/mL allowed DNA digestion and consequently an important cost reduction compared to 10 U/mL.

- Addition of Benzonase® endonuclease to the process improved the capacity of a Millistak+® HC C0HC clarification filter. Similarly, performance of TFF concentration was improved in the presence of Benzonase® endonuclease. During this same timeframe, a concentration factor of 14.4 was achieved in the presence of Benzonase® endonuclease as compared to 11 without.
- Most importantly, the addition of Benzonase® endonuclease reduced the level of Vero host cell DNA impurities from 25810 pg/mL (total amount removed) to less than 30 pg/mL. Benzonase® endonuclease is an effective tool for impurity clearance in vaccine processing and offers opportunities for improved efficiency of different processing steps.

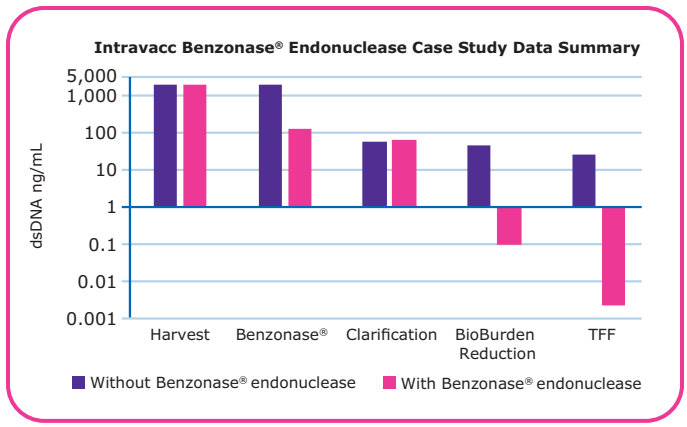


Figure 8. Case study data summary showing effective reduction of DNA by Benzonase® endonuclease.

- Overall, these data confirm that Benzonase® endonuclease can achieve DNA clearance at levels described in WHO and FDA guidelines (less than 10 ng/dose and less than 200 base pairs in length). As a genetically engineered endonuclease, this product is unique and demonstrated to be the most effective biochemical method for DNA removal.

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