AAV process intensification using high salt lysis & Benzonase® Salt Tolerant endonuclease

Agnieszka Lass-Napiorkowska[1], Christina Toenjes[1], Scott Hauser[1], Michelle P. Zoeller[2], Alexa Prager[2], Angeles Mecate-Zambrano[2], Sarah Lechat[3], Dmitry Zabezhinsky[4]

1- Upstream & Process Materials R&D, Process Solutions, Merck
2- Liquid Formulation R&D, Process Solutions, Merck
3- Strategic Marketing Management, Process Solutions, Merck
4- Upstream & Process Materials R&D, Process Solutions, Merck

Introduction

The overall process yield of adeno-associated virus (AAV) manufacturing is a major concern for biotherapeutic manufacturers for two major reasons:

- Viral vector yield can vary greatly with different process conditions
- Aggregation is a major pain-point affecting the recovery of viral particles

Commonly used buffers for cell lysis, a critical midstream step, contain physiological salt concentrations of ~150mM NaCl. In this study, we investigated the impact of lysis buffer with higher salt concentrations on AAV yield and infectivity.

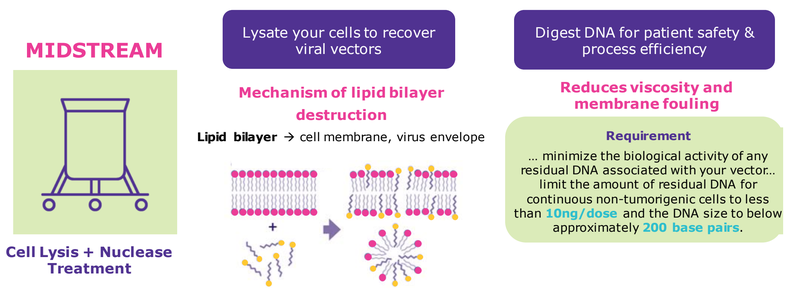
Cell lysis is a critical step during AAV production, and detergents are widely used for this purpose in biotherapeutic production processes.

Key challenges related to cell lysis include:

- Availability of efficient and sustainable detergents
- Impact of the detergent on the viral particles
- Removal of the detergent during purification steps

The process of lysis, facilitated by the detergent's ability to interact with the lipid bilayer of cells, leads to the complete disruption of cellular membrane. This liberates the viral particles along with impurities such as cellular remnants and nucleic acids from the host cells.

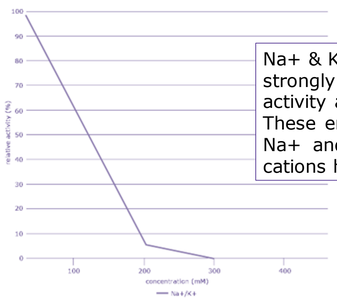
The combination of a detergent and an endonuclease from the Benzonase® portfolio to degrade these unwanted DNA fragments forms the **MIDSTREAM** unit operation.



Elimination of DNA from a drug product is critical for ensuring patient safety. Regulatory bodies such as the FDA* have established guidelines defining acceptable levels of residual DNA in the final dosage as part of good practice standards.

Source* Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)- Draft Guidance for Industry – FDA July 2018

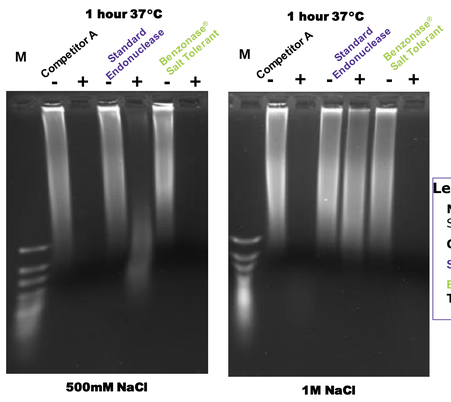
While standard endonuclease have a proven efficiency from 0 to 200mM concentrations, high ionic strengths hinder the interaction between the enzyme and DNA. The impact of salt concentrations above 200mM NaCl on nuclease activity thus warrants investigation.



Na+ & K+ inhibit standard endonuclease activity strongly leading to a complete depletion of activity at concentrations above 300 mM.
These enzymes exhibit an identical response to Na+ and K+, presuming all other monovalent cations have a similar effect.

To enable use of high salt concentrations during this midstream operation, we developed a new Benzonase® endonuclease using state-of-the-art protein engineering capabilities. **Benzonase® Salt Tolerant endonuclease** efficiently digests DNA at NaCl concentrations up to 1M.

The efficacy of DNA digestion under various salt concentrations was assessed by employing agarose gel electrophoresis. This allowed for the evaluation of the extent of DNA degradation using different salt-tolerant endonucleases, including Benzonase® Salt Tolerant endonuclease and standard endonucleases.



Legend
M – GeneRuler Ultra Low Range DNA Ladder Cat# SM1213
Comp A – Salt Active Nuclease - Competitor A
Standard Endonuclease – Cat# 103773 / Denarase®
Benzonase® Salt Tolerant– **Benzonase® Salt Tolerant Endonuclease Emprove® Expert**

Benzonase® Salt Tolerant endonuclease
⇒ Digests DNA completely at 1M salt.
⇒ Shows similar or better perfromances Vs Competitor A.

Nuclease selection

Key requirements for nuclease selection in AAV manufacturing

While a number of nucleases are commercially available, the lack of highly reliable supply chains and batch release claims present a problem for gene therapy developers.

The following list summarizes key criteria for enzyme selection for AAV manufacturing.

Key criteria for Enzyme selection for AAV manufacturing

1. IPEC PQG GMP or equivalent
2. >99% Pure product
3. Devoid of Post-translational modifications
4. Mycoplasma Test
5. Adventitious viruses Test
6. Endotoxin Test
7. Tailgate samples availability
8. Strong logistic & Supply Robustness
9. Technical support for application related questions
10. Robust detection method

The **Benzonase® Salt Tolerant endonuclease Emprove® Expert** meets these different requirement as outlined in the table below.

Features/Applications	Benzonase® Salt Tolerant Endonuclease Emprove® Expert
Catalogue code	1.04445
Origin	Proprietary protein engineering platform
Post-translational modifications	No. well defined protein profile, full batch to batch reproducibility
Purity	99%
IPEC-PQG GMP	Yes
Monovalent Cation concentration (Na+, K+...)	**200mM-1M Effective*** **300mM-600mM Optimal***
FDA Master File (BBMF or DMF) available & Emprove® Expert Dossiers	Emprove® Expert Dossiers available, FDA DMF available through 2024.
Non-Animal-Origin (NAO), recombinant from E.Coli in chemically defined medium	Yes
Shipment with temperature strips	Yes
Lot release test adventitious viruses (3 cell lines)	Available as a custom option
Mycoplasma test	Yes
Tailgate samples	Yes (with 4M unit size)
Endotoxins (LAL) microbial testing	< 0.25 EU/1,000 U < 10 CFU/100,000 U
Supply robustness	Two redundant production sites
Detection method	ELISA Available through 2024

** "Optimal" is defined as the condition under which Benzonase® endonuclease retains > 90% of its activity.*

** "Effective" is defined as the condition under which Benzonase® endonuclease retains > 15% of its activity*

More information on this **Emprove® Expert GMP (IPEC-PQG)** enzyme can be found in the product brochure.

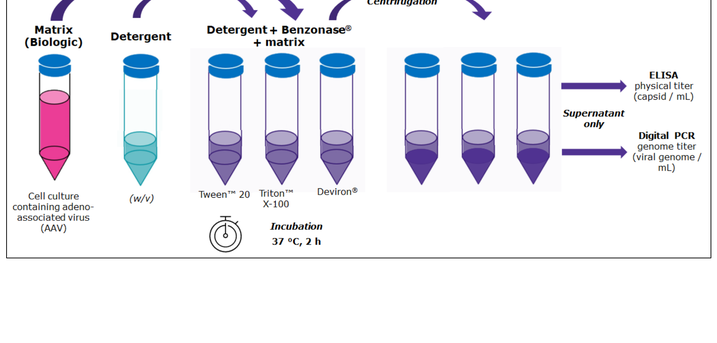
High Salt Lysis Performance

Method

HEK293 and Sf-RVN® cells were cultivated in the appropriate cell culture media. HEK293 cells were transfected with the plasmids of interest using polyethylenimine (PEI) reagent. Sf-RVN® cells were infected with Baculovirus at the time of seeding. At the end of the cultivation process, the cells were lysed using the following buffer:

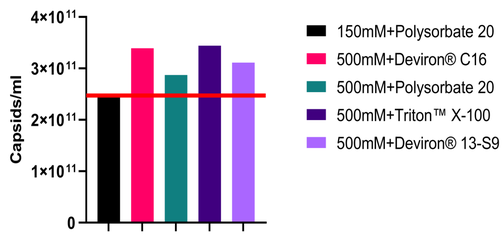
- **Detergent: 0.5 % wt.**
- **Nuclease: 25 U/mL Benzonase® Salt Tolerant endonuclease** with 2 mM $MgCl_2$
- Lysis time: 2 h
- Temperature: 37 °C

After lysis, virus-containing supernatant was clarified by centrifugation. The supernatant was analyzed for the number of viral capsids by ELISA and viral genome copies by dPCR. Following detergent removal, the AAV infectivity was measured. Efficient DNA removal was assessed using nanodrop and agarose gel electrophoresis



AAV5 titers increased by an average of 29% upon lysis with high salt

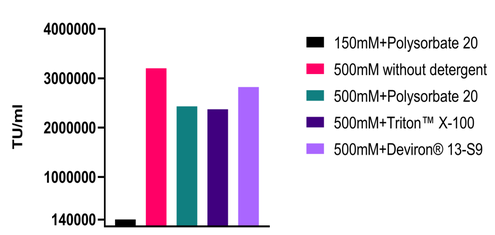
AAV vector titers (as measured by ELISA) were compared upon lysis at 150 and 500mM sodium chloride (NaCl) with four commonly used cell lysis detergents. With 500mM NaCl and for all detergents tested, capsid titer was on average 29% higher than with 150mM NaCl.



Baseline: 150mM NaCl and Polysorbate 20	Efficiency compared to baseline
500mM NaCl + Polysorbate 20	**+16%**
500mM NaCl + TRITON™ X-100	**+39%**
500mM NaCl + Deviron® C16	**+37%**
500mM NaCl + Deviron® 13-S9	**+25%**

AAV2 infectivity was increased by an average of 1900% upon lysis with high salt

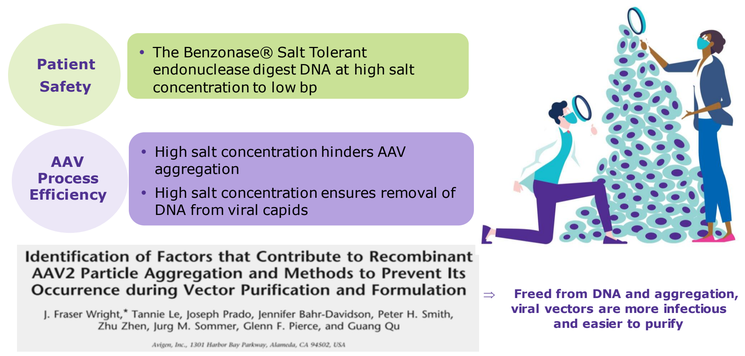
AAV infectivity, a measure of potency, was assessed using the same experiment with an AAV2 serotype. Upon lysis with 500mM NaCl, AAV2 infectivity was on average 1900% higher compared to lysis with 150mM NaCl and was independent of the detergent used.



Baseline: 150mM NaCl and Polysorbate 20	Efficiency compared to baseline
500mM NaCl + Polysorbate 20	**+1700%**
500mM NaCl + TRITON™ X-100	**+2200%**
500mM NaCl + Deviron® C16	**+1700%**
500mM NaCl + Deviron® 13-S9	**+2000%**

Discussion

Although the precise mechanism is not fully elucidated, it is evident that the concentration of NaCl has a significant impact on viral vector production. The primary hypothesis centers on the fact that high ionic strength can impede the aggregation of AAV, thereby facilitating the release of the host cell DNA for the viral capsids.



Freed from DNA and aggregation, viral vectors are likely to be more infectious.
As standard endonucleases are inactive at salt concentrations above 200mM, the use of **Benzonase® Salt Tolerant endonuclease** is needed to ensure reliable nucleic acid removal and address regulatory requirements for patient safety.

Conclusion

1 **Lysis with High Salt Increases Viral Titer**
500mM NaCl increases viral titer by 29% in AAV5

2 **Lysis with High Salt Increases Viral Infectivity**
500mM NaCl increases viral infectivity by >1500% in AAV2

High Salt Lysis with Benzonase® Salt Tolerant Endonuclease

3 **Benzonase® Salt Tolerant Endonuclease** efficiently digest DNA at high salt concentrations across all tested detergents

4 **Choose the right nuclease**
- Emprove® Expert
- IPEC PQG GMP
- No glycosylations

5 **Recommendation**
Process development is needed for every new AAV production – detergent and salt concentration must be tested beforehand