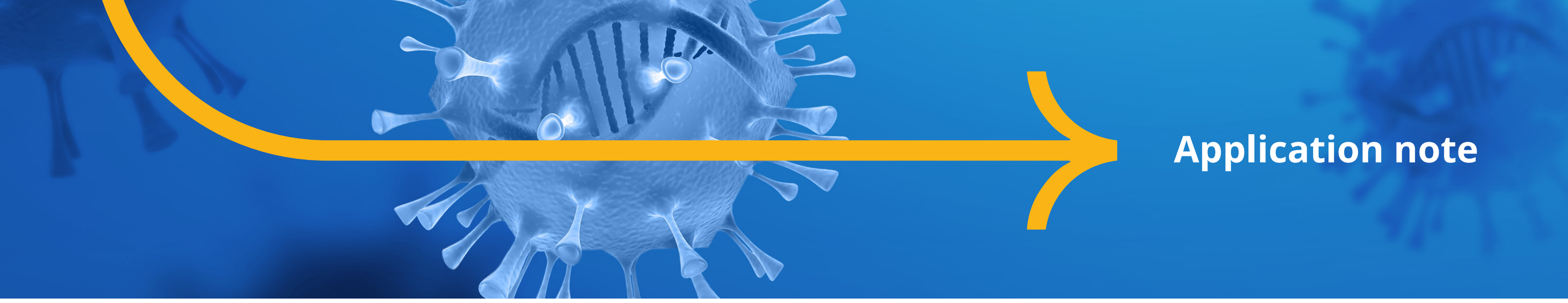


Recombinant Insulin Improves AAV2 Yield and Encapsidation in HEK293 Suspension Production

AAV vectors are widely used in gene therapy trials, but manufacturing scale-up has not kept pace with the clinical demand. To address this bottleneck, we tested Recombinant Insulin supplementation in HEK293 suspension cultures for producing viral vectors. Our results show improved AAV2 yields and encapsidation in supplemented cultures, with no adverse impact on product quality.

Introduction

Adeno-associated virus (AAV) vectors are central to modern gene therapy because they combine efficient gene delivery with a favorable safety record.[1] Dozens of AAV-based programs are advancing through clinical development, creating a growing demand for scalable, high-quality vector production.[2] However, large-scale manufacturing remains a significant bottleneck, with process scientists striving to deliver high yields of genome-containing capsids under reproducible and regulatory-compliant conditions.

Several factors make this difficult. Transient transfection of HEK293 cells, the most common AAV production system, is inherently variable and difficult to scale.[3] Yields are often limited by a low proportion of genome-containing particles, which increases downstream purification burden. Batch-to-batch variability complicates process control, while the need to meet regulatory expectations for consistency and comparability adds further pressure.

Common media optimization strategies add another layer of constraint. Animal-derived components such as fetal bovine serum (FBS) raise regulatory concerns.[4] However, chemically defined premixes restrict flexibility by locking researchers into fixed formulations. These limitations highlight the need for supplements that are animal-free, consistent, and adjustable.

Recombinant Insulin is a well-established media supplement that supports cell metabolism and productivity.[5] By adjusting insulin addition independently, scientists can optimize a key parameter without relying on animal-derived components or losing process flexibility.

In this study, we assessed Recombinant Insulin (also known as Insulin Human AF) as a supplement for HEK293 suspension production of AAV2, a widely studied serotype used in both basic research and early clinical development. We evaluated the concentration and timing of addition relative to transfection, and analyzed the impacts on vector yield, reproducibility, encapsidation, and downstream quality attributes.

Study Overview

We performed three HEK293 suspension culture runs using a three-plasmid AAV2 production system at 1 L scale. Run 1 established the suitable conditions for further testing. Runs 2 and 3 were carried out to validate the optimal conditions established in Run 1. Across all runs, AAV titers, cell growth and viability, encapsidation efficiency, and key product quality attributes – including purity, stability, and potency – were evaluated in supernatant, cell pellet, combined harvest, and clarified bulk harvest using established analytical methods.

Materials and Methods

Insulin supplementation

Recombinant Insulin (Insulin Human AF, Novo Nordisk Pharmatech) was added at 0, 5, 10, or 20 mg/L at four time points relative to transfection (24 h before, 2 h before, at transfection, or 4 h after) in Run 1. Runs 2 and 3 were carried out under the selected Run 1 conditions, with insulin-supplemented and control cultures included.

Cell culture

Serum-free adapted Freestyle 293F (HEK293) suspension cells were maintained in F17 medium supplemented with 8 mM L-glutamine and 0.1% Poloxamer 188. Cultures were incubated at 37 °C, 8% CO_2, and 125 rpm in 1 L shake flasks. Cells were seeded at 0.8 × 10^6 cells/mL with >98% viability at inoculation.

Plasmids and transfection

A three-plasmid system was used for AAV2 production, consisting of:

- pALD-ITR-WPRE-GFP transfer plasmid
- Rep-Cap2 plasmid
- Helper plasmid

Plasmids were purified using standard endotoxin-free kits. Transfection was performed with linear PEI (40 kDa) at a DNA:PEI ratio of 1:3, using 1 µg DNA per 10^6 cells. DNA–PEI complexes were prepared in Opti-MEM, incubated for 25 min, and added to cultures.

Sampling and harvest

Cell density and viability were measured at seeding, at transfection, and at 24 h and 48 h post-transfection. Cultures were harvested 48 h post-transfection. Small-volume samples were collected for titer analysis, and the remaining harvest was used for AAV purification. Clarified bulk harvest samples were prepared by lysing the harvested cultures, treating with benzonase, centrifuging, and filtering (0.2 µm) to provide clarified material for CP and VG titer analysis.

Titer determination

Capsid protein (CP) titers were measured in pellet, supernatant, whole harvest, and purified samples using a commercial AAV2 ELISA kit. Clarified harvest samples were corrected for dilution introduced during processing. Vector genome (VG) titers were determined by qPCR using WPRE-specific primers. Standard curves were generated from linearized GFP plasmid DNA.

Encapsidation analysis

Encapsidation efficiency was calculated as the VG/CP ratio at the clarified harvest stage. For purified samples, full and empty capsids were separated and quantified by AEX-HPLC.

Purity assessment

Purity of virus preparations was assessed by SEC-HPLC. All runs showed >99% monomer content. Protein identity was confirmed by SDS-PAGE and Western blot, with VP1, VP2, and VP3 detected at expected molecular weights.

Potency assay

HEK293T cells were seeded in 96-well plates and transduced with serial dilutions of purified AAV2. After 48 h, GFP expression was analyzed by flow cytometry. Transduction efficiency was reported as % GFP-positive cells.

Evaluation of Insulin Timing and Concentration

To identify the optimal supplementation condition, Run 1 compared insulin addition at four time points (24 h before, 2 h before, at transfection, and 4 h after) and three concentrations (5, 10, and 20 mg/L) against a no-insulin control.

Capsid protein (CP) titers were variable across the different conditions, but Batch 2 – where insulin was added 2 h before transfection – showed the most consistent increases across supernatant, cell pellet, and total harvest samples compared to the control without insulin (Figure 1). Vector genome (VG) titers followed the same pattern, with Batch 2 demonstrating reliable improvements across all insulin concentrations (Figure 2). Together, these titer measurements highlighted Batch 2 as the most promising condition.

Cell growth and viability provided additional support for this conclusion. At 48 h post-transfection, modest increases in viable cell counts were observed in batches supplemented 24 h or 2 h before transfection, suggesting a positive impact on culture health under these conditions (Figure 3).

The combination of titer measurements and cell count trends indicated that adding 10 or 20 mg/L insulin two hours before transfection gave the most consistent benefit, which was then advanced into Runs 2 and 3 for reproducibility testing.

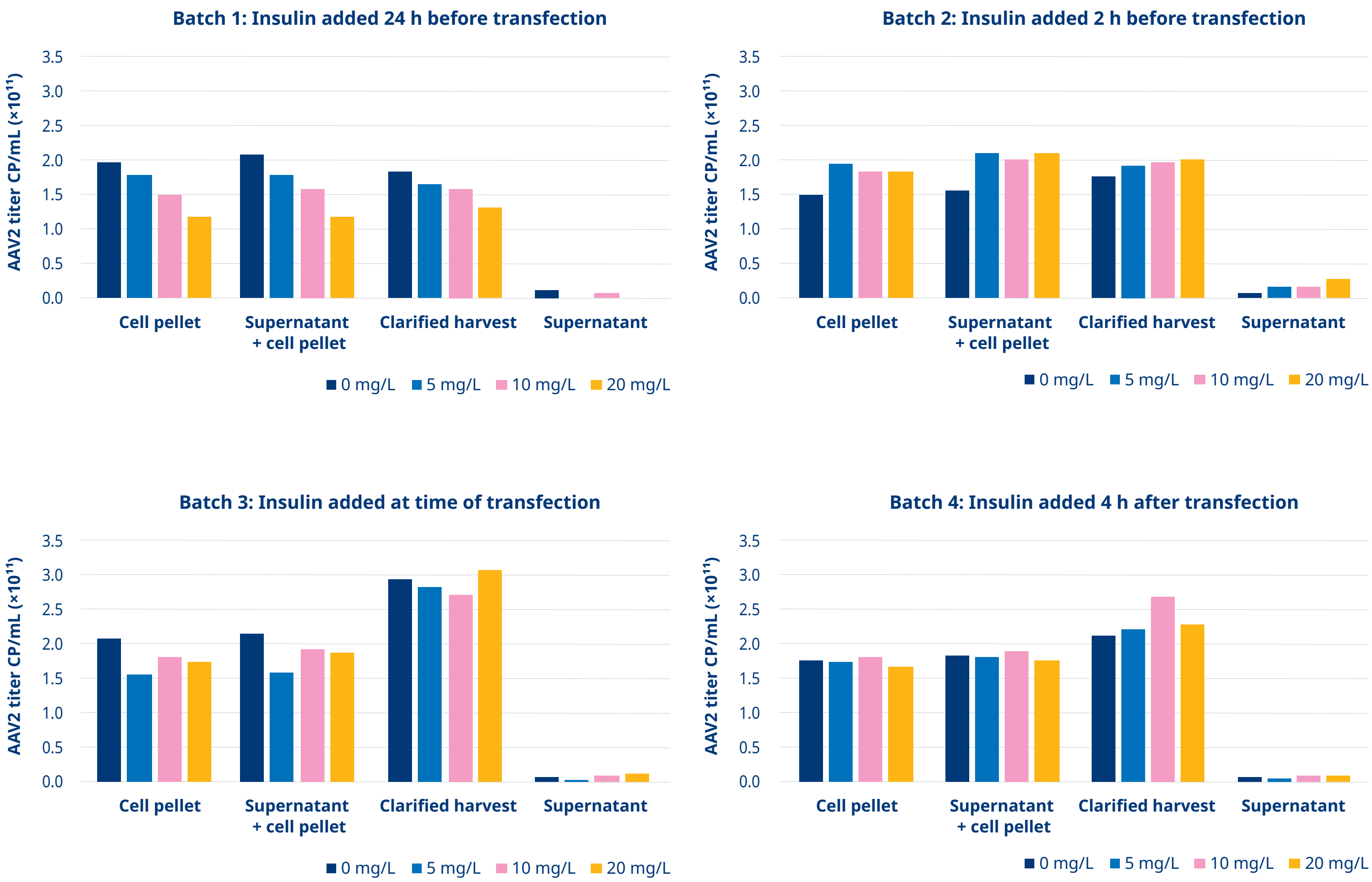


Figure 1: Insulin enhanced CP titers when added 2 hours before transfection. CP titers were measured in supernatant, cell pellet, and clarified total harvest samples using an AAV2-specific ELISA.

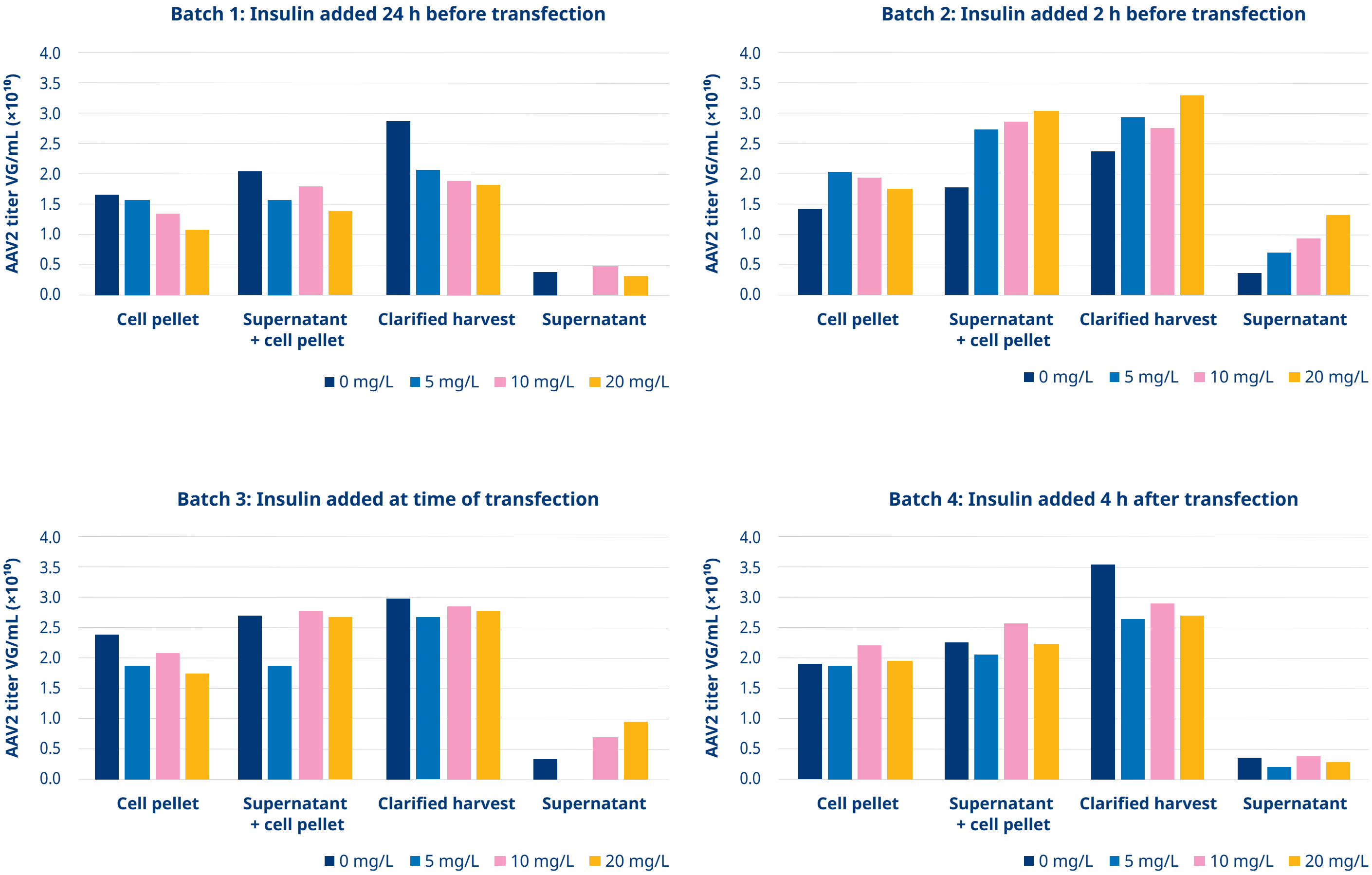


Figure 2: Insulin improved VG titers when added 2 hours before transfection. VG titers were quantified by qPCR in supernatant, cell pellet, and clarified total harvest samples.

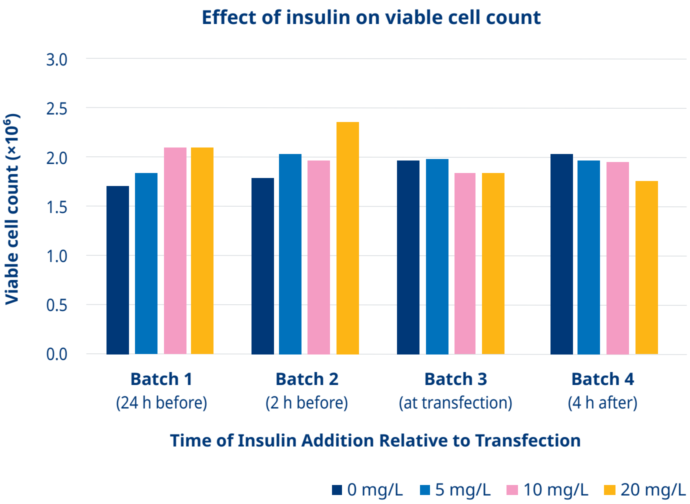


Figure 3: Insulin increased viable cell counts in selected conditions. Viable cell counts were measured 48 hours after transfection across four batches, each with insulin added at a different time point relative to transfection.

Insulin Increases AAV Titers and Productivity

Achieving high and reproducible AAV titers is a top priority in process development. Based on the Run 1 screening, insulin addition at 10 and 20 mg/L two hours before transfection was advanced for confirmation in subsequent 1 L runs. Under these conditions, insulin increased both CP and VG titers compared to the control without insulin (Figure 4). Viral titers were measured in different process fractions, including the harvest supernatant, where AAV concentrations are typically lower than in the cell-associated fraction. Nevertheless, even in the supernatant, insulin supplementation led to increased titers. These improvements highlight the potential of insulin supplementation to support higher volumetric productivity and more consistent process performance.

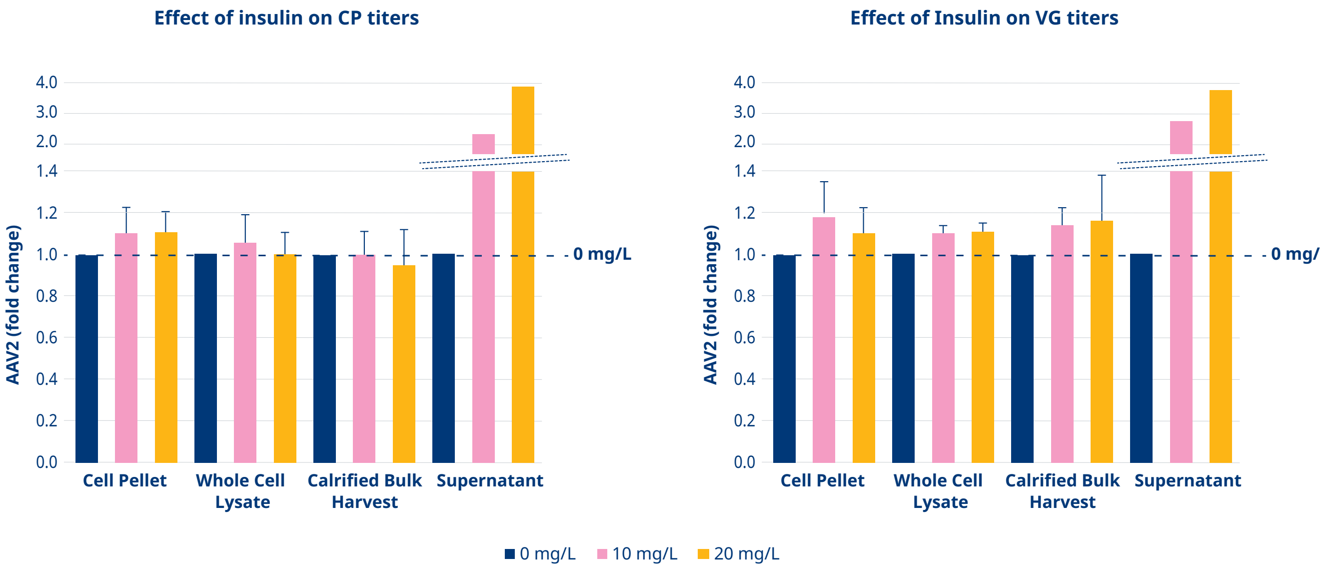


Figure 4: Addition of Recombinant Insulin increased CP and VG titers across runs. Data represent three independent runs; error bars indicate standard deviation. Supernatant values are from one run, as VG control titers in other runs were below the quantification limit and could not be normalized.

Insulin Improves Encapsidation Efficiency at Harvest

Maximizing the proportion of full, therapeutically active vectors is essential for effective AAV manufacturing. To evaluate whether insulin supplementation influenced encapsidation (the process of packaging vector genomes into capsids), we determined the VG/CP ratio at the clarified harvest stage. Our results show that Recombinant Insulin supplementation provided a measurable advantage: across the three runs, encapsidation efficiency increased from ~12% in controls to ~14% in insulin-supplemented cultures. This corresponded to a 1.17-fold increase at 10 mg/L and a 1.25-fold increase at 20 mg/L (Figure 5).

These findings indicate that insulin increased the proportion of genome-containing particles at harvest. Starting downstream purification with a higher proportion of full capsids reduces the purification burden and provides a wider processing window to achieve final purity targets.

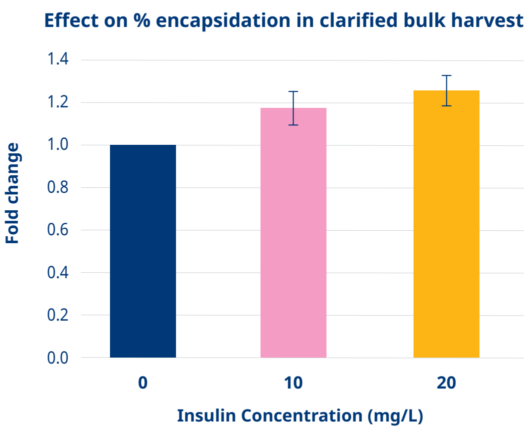


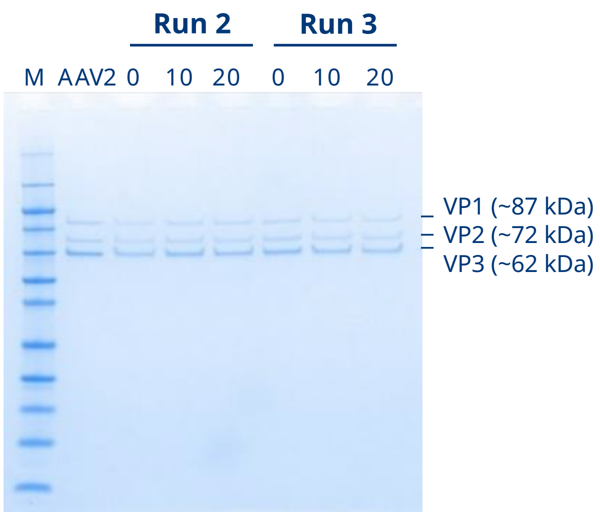
Figure 5: Insulin supplementation increased relative encapsidation at harvest. Data are shown as fold change in % encapsidation (VG/CP) in clarified bulk harvest, normalized to the control condition. Insulin supplementation increased the encapsidation ratio relative to control, indicating improved genome packaging. Error bars represent standard deviation across three runs.

Capsid Protein Profile Is Unchanged

We performed SDS-PAGE and Western blot analysis to examine the capsid protein profiles. Our results showed that the expected VP1, VP2, and VP3 bands, with no detectable impurities, are present in AAV2 purified from the insulin-supplemented cultures (Figure 6), confirming product identity and purity. Preserving capsid composition is essential for regulatory acceptance and ensures comparability with established production standards.

Virus Stability Is Maintained and Aggregation Minimized

To determine whether supplementation with Recombinant Insulin influences the aggregation behavior, we analyzed the purified AAV2 preparations by SEC-HPLC. The results show a monomer content of greater than 99% across all batches (Figure 7), indicating no increase in aggregation risk. Low aggregation is essential for minimizing immunogenicity and ensuring long-term stability during storage and distribution.



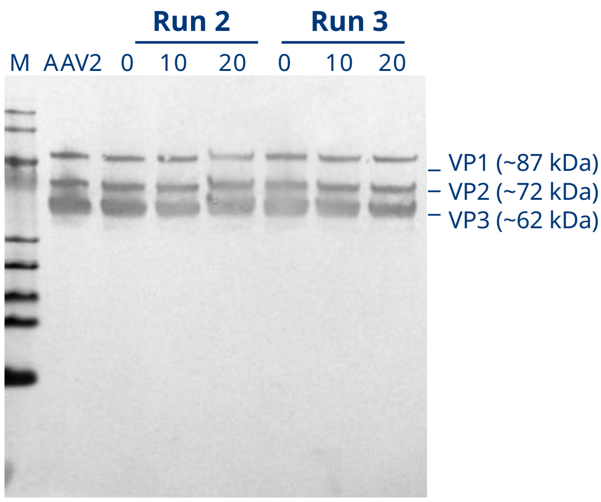


Figure 6: Capsid protein profile is unchanged by insulin supplementation. SDS-PAGE and Western blot confirmed the expected VP1, VP2, and VP3 bands with no detectable impurities, demonstrating that capsid composition was unaffected. Samples: purified empty AAV2 and purified AAV2 from two production runs (Run 2 and Run 3) with insulin added 2 h before transfection at 0, 10, or 20 mg/L.

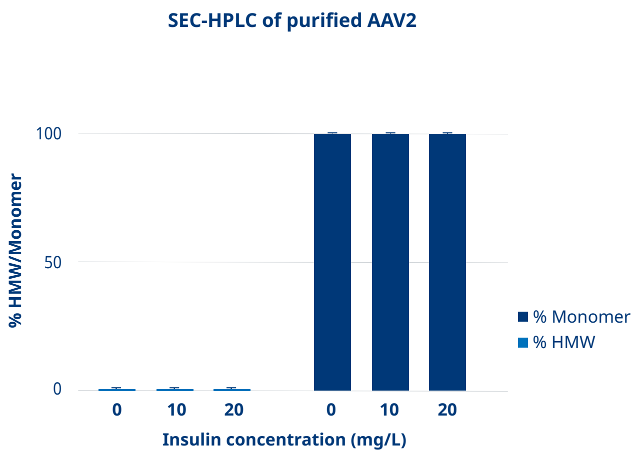


Figure 7: Insulin supplementation does not affect stability or aggregation. SEC-HPLC analysis showed >99% monomer content across all runs, confirming no adverse effect on aggregation or stability.

Functional Potency Is Maintained

To confirm that insulin supplementation did not compromise the vector potency, we tested the harvest supernatant collected 48 h post-transfection in a cell-based transduction assay. In these experiments, vectors from insulin-supplemented runs produced an apparent dose-dependent increase in GFP expression (Figure 8). The increase in upstream yield was achieved without compromising biological activity, showing that vector potency was maintained.

A trend toward higher potency was observed at 20 mg/L insulin, which may reflect the increased presence of functional viral particles in the supernatant – consistent with the elevated VG titers seen at this concentration. Since mature AAV particles are known to accumulate in the supernatant during production, these findings suggest that insulin may enhance the release or production of functional vector.

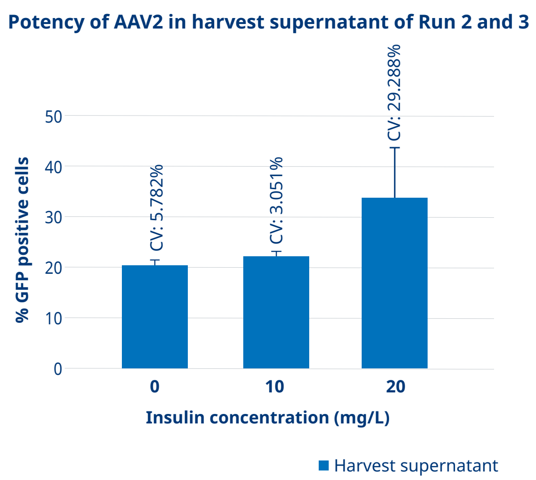


Figure 8: Vector potency is maintained with insulin supplementation. Potency of AAV2 in harvest supernatants from Runs 2 and 3 was assessed by transducing HEK293T cells and measuring GFP expression as a readout of functional vector delivery. Bars show the percentage of GFP-positive cells at increasing doses, confirming that biological activity was preserved.

Insulin Supports Healthy Cell Growth and Viability

At 48 hours post-transfection, cultures supplemented with insulin showed slightly higher viable cell counts compared to controls (Figure 9). While the increase was modest, it was repeatable across runs and accompanied by a more pronounced rise in viral titers, suggesting insulin may play a role in activating intracellular pathways that support virus production. The consistently high viability at harvest suggests that cultures could potentially be maintained longer, providing additional flexibility in process scheduling and the possibility of further increasing viral yield with extended culture duration.

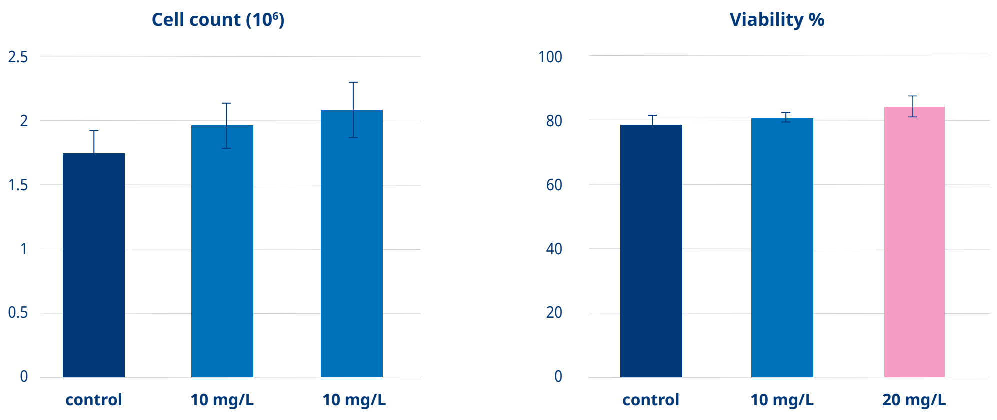


Figure 9: Effect of insulin on viable cell count and cell viability at 48 h post transfection. Viable cell counts and % viability were measured across three runs at the 48-hour harvest point. Insulin-supplemented cultures showed higher viable cell numbers and maintained high viability compared to controls.

Potential Operational Benefits of Insulin Supplementation

The improved AAV titers observed with insulin supplementation translate directly into process efficiencies that matter in biomanufacturing. As shown in Table 1, a modest 1.2-fold increase in AAV yield would reduce the required bioreactor volume by 17% (e.g., from 80,000 L to 66,600 L to produce 400 doses). For manufacturers, this reduction in volume can help ease capacity constraints, lower raw material consumption, and free up valuable production time – all of which support more flexible, cost-efficient operations.

Bioreactor Scale	200 L			
Number of doses needed	400			
Average dose/patient	1E+15			
Typical yield (after purification) VG/L	0.5E+13			
Yield improvement	**Baseline w/o insulin**	**1.1**	**1.2**	**1.4**
Liters needed for 400 doses	80.000	72.700	66.600	57.100
Reduction in bioreactor volumes with insulin		9%	17%	29%

Table 1: Modest titer improvements translate into substantial operational benefits. A 1.2-fold improvement lowers production volumes by up to 17%, easing capacity constraints and supporting more efficient AAV manufacturing. Calculations assume an average dose of 1×10^{15} VG and a purified yield of 0.5×10^{13} VG/L from a 200 L culture. We estimated the bioreactor volume needed to produce 400 doses at baseline and under three insulin-boosted yield scenarios.

Conclusion

Recombinant Insulin supplementation increased AAV2 titers and improved genome encapsidation in HEK293 suspension cultures without affecting downstream quality attributes. Across three independent runs, supplementation at 10 and 20 mg/L two hours before transfection reproducibly increased CP and VG titers, while also improving encapsidation efficiency at harvest. Importantly, capsid composition, stability, and potency were maintained, confirming that product quality was not compromised.

In addition to these upstream and product quality gains, insulin supplementation supported healthy cultures at harvest, with modestly increased viable cell counts and consistently strong viability across replicates. This suggests that insulin contributes to a stable and resilient production environment.

The high viability at 48 h post-transfection raises the possibility of extending the culture duration beyond standard harvest times, potentially increasing viral yields further while providing greater flexibility in process scheduling.

From a manufacturing perspective, even modest titer increases (for example, 1.2-fold) can translate into substantial reductions in the bioreactor volumes required for production. This underscores the practical manufacturing value of insulin supplementation, extending its impact beyond yield gains to overall efficiency and scalability.

Together, these findings highlight Recombinant Insulin as a simple, animal-free supplement that enhances reproducibility and scalability in AAV vector manufacturing, while offering potential avenues for future process intensification.

Key Takeaways

Recombinant Insulin supplementation improved both yields and process performance, offering tangible benefits for AAV manufacturing:

- Recombinant insulin increased AAV2 titers, improving volumetric productivity.
- Encapsidation efficiency improved, with a higher proportion of genome-containing capsids, easing downstream purification.
- Regulatory-critical attributes were maintained, with no loss of product quality or potency.
- Insulin supported process advantages, including healthy cultures, extended harvest potential, and lower reactor volume needs.

Recombinant Insulin for Reliable AAV Manufacturing

With decades of experience supplying high-quality raw materials to the biopharmaceutical industry, Novo Nordisk Pharmatech supports the development of robust and compliant manufacturing processes for biologics and advanced therapies, including viral vector–based gene therapies.

Our Recombinant Insulin (Insulin Human AF) is produced to the highest cGMP standards and is supplied with complete documentation and regulatory support. As an animal-free, consistent, and scalable supplement, it contributes to batch-to-batch consistency and reliable process performance.

References

1. Moldavskii D, Gilazieva Z, Fattakhova A, *et al*. AAV-Based Gene Therapy: Opportunities, Risks, and Scale-Up Strategies. *Int J Mol Sci*. 2025;26(17):8282. doi:10.3390/ijms26178282
2. Kuzmin DA, Shutova M V., Johnston NR, *et al*. The clinical landscape for AAV gene therapies. *Nat Rev Drug Discov*. 2021;20(3):173-174. doi:10.1038/d41573-021-00017-7
3. Blessing D, Vachey G, Pythoud C, *et al*. Scalable Production of AAV Vectors in Orbitally Shaken HEK293 Cells. *Mol Ther Methods Clin Dev*. 2019;13:14-26. doi:10.1016/j.omtm.2018.11.004
4. Weber T, Malakpour-Permlid A, Chary A, *et al*. Fetal bovine serum: how to leave it behind in the pursuit of more reliable science. *Frontiers in Toxicology.Frontiers Media* SA. 2025;7. doi:10.3389/ftox.2025.1612903
5. Barnes D, Sato G. Serum-free cell culture: a unifying approach. *Cell*. 1980;22(3):649-655. doi:10.1016/0092-8674(80)90540-1

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