# AAV-Nanobody manufacturing including a chromatography purification step with protein A.

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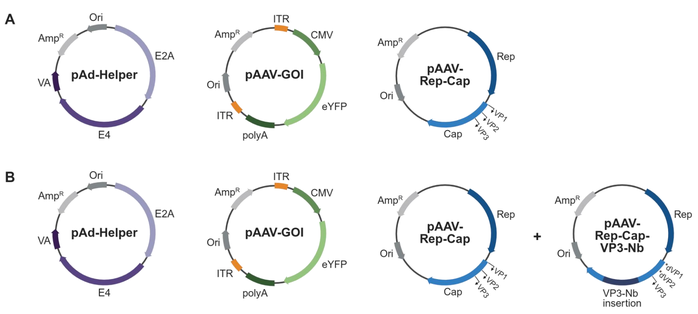
## Abstract

AAV vectors are utilized for a broad range of disease treatments in gene therapy. After an initial surge of therapeutic development based on natural AAV serotypes, the scientific community has been exploring options to further improve AAV-based vectors and possibilities to redirect capsids to specific cells and tissues of interest. The AAV capsid consists of three viral proteins, VP1, VP2 and VP3, which tolerate the insertion of small peptides or larger protein fragments into the surface-exposed variable loop regions. This feature can be exploited to rationally design AAVs with re-targeting properties. One approach is the use of single variable domains from camelid heavy chain antibodies (VHH or nanobodies) expressed as fusion proteins with viral capsid components and thus being incorporated into the assembled particles.

Most attempts at generating nanobody-decorated AAVs aim at incorporation within VP1 [1,2] or, less frequently, VP2 [3,4] proteins. We have generated a set of AAV-Nanobody (AAV-Nb) delivery vectors with a novel proprietary design based on an insertion site in the viral protein VP3 [patent pending, 5]. Concurrently, we have established a new manufacturing protocol for the production and purification of AAV-VP3-Nb vectors. Our process requires co-transfection of four plasmids in a suspension cell line, resulting in assembled capsids which maintain their VP1, VP2 and VP3 composition while displaying 4-8 VP3-nanobody units on the capsid surface. DSP includes an affinity chromatography step with Protein A columns, an iodixanol gradient for the enrichment of full particles and buffer exchange for final sample formulation.

Our industry-grade protocol for AAV-VP3-Nb production is scalable and universally applicable, irrespective of the capsid serotype incorporating the nanobody and the specificity of the nanobody to its target, due to the interaction of protein A with a protein A-binding motif in the nanobody scaffold. The yields are comparable to the purification of AAV-Nb capsids via AAV-specific affinity chromatography. Endotoxin levels of final batches are consistently below the 1.00 EU/mL threshold, enabling the use of produced AAV-Nbs for *in vivo* animal studies. Quality control of AAV-Nb lots by PAGE showed high purity across all productions and confirmed the incorporation of VP3-Nb protein units within the capsids, as also supported by mass spectrometry analysis. We further confirmed the biological functionality of AAV-Nbs in a HEK293 cell-based assay and showed that re-targeting occurs via the incorporated nanobody moiety. The established protocol generates robust results with consistent titers across different batches and reproducible yields between 1E+12 and 2E+12 viral genome (vg) units purified from 120 mL suspension cell volume. We have monitored all manufacturing steps with in-process controls (IPCs) and identified focus areas for further improvement, e.g., tailoring the transfection parameters to the AAV-Nb technology or further optimizing elution buffer settings.

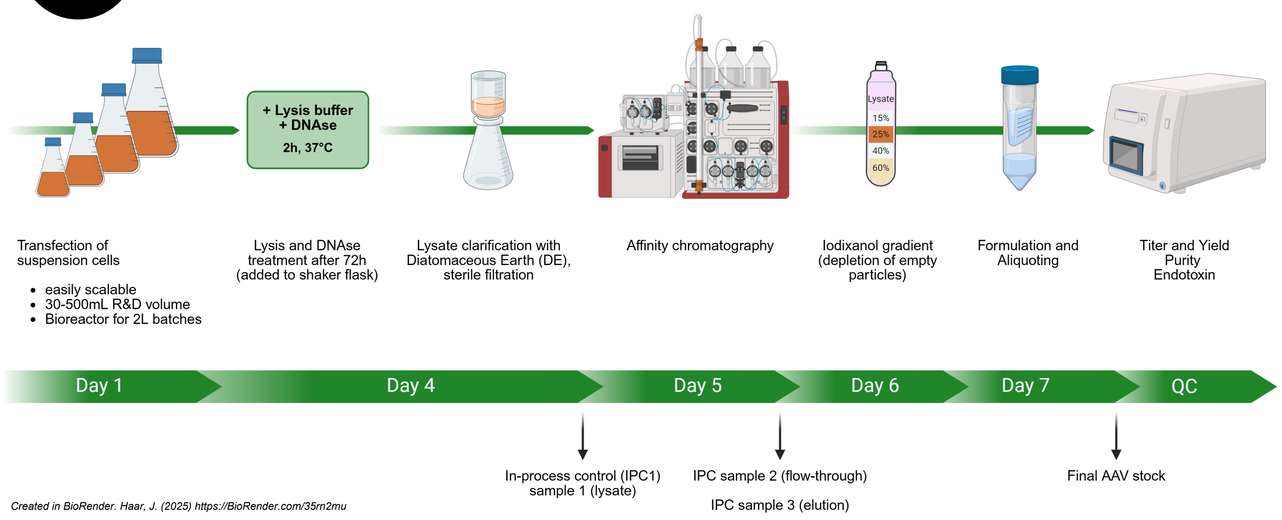
## 1 Plasmid design for transfection




*Created in BioRender. Haar, J. (2025) https://BioRender.com/in63pdl*

*Plasmids used for transient transfection in AAV USP of AAV9 (upper panel A) and AAV9-VP3-Nb particles (lower panel B). Co-transfection for AAV-VP3-Nb manufacturing was performed at a fixed Rep-Cap plasmid ratio of 4:1 with additional evaluation of optimal conditions explored by DoE (data not shown).*

## 2 Overview of main DSP steps for AAV-Nb manufacturing

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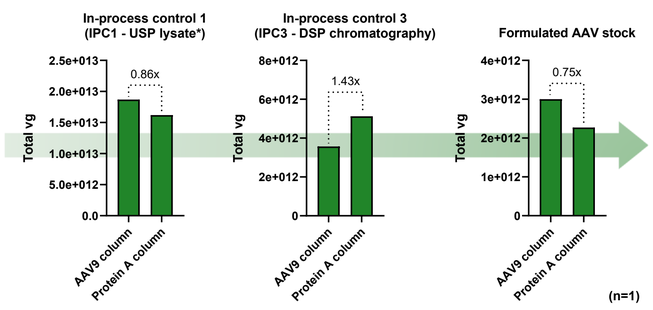
*Manufacturing of AAV-Nbs is performed via transfection of suspension cells enabling scalability. DSP includes an affinity chromatography step with Protein A columns, a subsequent iodixanol gradient for the enrichment of full particles and buffer exchange for final sample formulation. The established process is compatible with our ISO-9001:2015 certified SOPs for AAV manufacturing.*

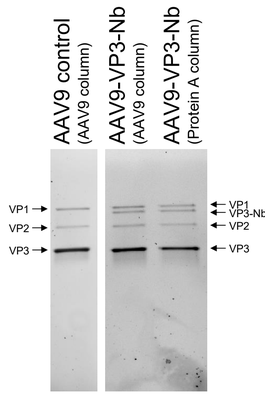
## 3 Yields from purification by AAV9 or Nb-positive selection are equivalent




*Purifications performed starting with the same lysate, value differences based on titration of 2 aliquots

*Purification of the same AAV9-VP3-Nb lysate using AAV9 columns or Protein A columns for affinity selection via capsid or nanobody binding. Efficiency of the chromatography step (IPC3) is slightly improved with Protein A columns. Final recovery for AAV9-VP3-Nb is comparable with about 14-16% of lysate particles present after formulation.*

## 4 Incorporation rates of VP3-Nb units are comparable for both purification methods



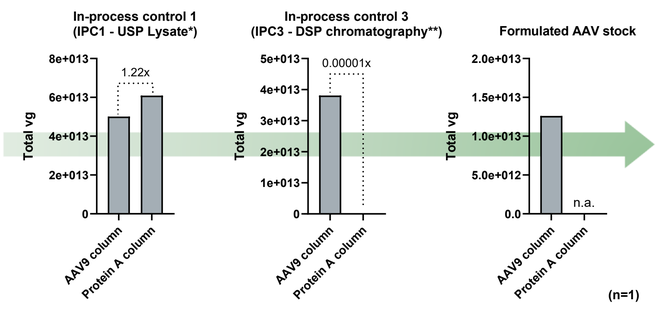

| % Band intensity | AAV9 (AAV9 column) | AAV9-VP3-Nb (AAV9 column) | AAV9-VP3-Nb (Protein A column) |
|---|---|---|---|
| VP1 [%] | 11.58 | 13.09 | 14.22 |
| **VP3-Nb [%]** | **0** | **11.47** | **13.31** |
| VP2 [%] | 6.89 | 10.56 | 11.19 |
| VP3 [%] | 81.53 | 64.88 | 61.29 |
| VP3 total [%] | 81.53 | 76.35 | 74.6 |
| Incorporated VP3-Nb [% of total VP3] | 0.00 | 15.02 | 17.84 |
| **Estimated number of VP3-Nb units per AAV** | | | |
| VP1 number | 7 | 8 | 9 |
| **VP3-Nb number** | **0** | **7** | **8** |
| VP2 number | 4 | 6 | 7 |
| VP3 number | 49 | 39 | 37 |
| Total | 60 | 60 | 60 |

*Table 1: Quantification of VP1, 2 and 3 protein bands and estimated number of displayed VP3-Nb units per AAV.*

| Viral particles with X VP3-Nb units | Theoretical abundance | | |
|---|---|---|---|
| | AAV9 (AAV9 column) | AAV9-VP3-Nb (AAV9 column) | AAV9-VP3-Nb (Protein A column) |
| 0 | **100.0%** | 2.6% | 1.2% |
| 1 | 0.0% | 9.7% | 5.5% |
| 2 | 0.0% | 18.1% | 12.5% |
| 3 | 0.0% | **22.1%** | 18.5% |
| 4 | 0.0% | 19.7% | **20.2%** |
| 5 | 0.0% | 13.8% | 17.3% |
| 6 | 0.0% | 7.9% | 12.0% |
| 7 | 0.0% | 3.8% | 7.0% |
| 8 | 0.0% | 1.5% | 3.5% |
| 9 | 0.0% | 0.5% | 1.5% |
| 10 | 0.0% | 0.2% | 0.6% |

*Table 2: Distribution of VP3-Nb incorporation as determined by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS).*

## 5 AAV9 particles are not purified via Protein A columns



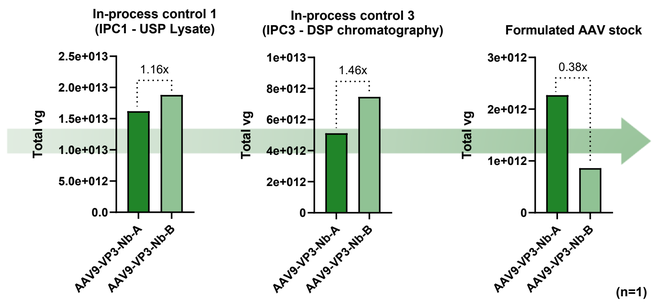

*Purifications performed starting with the same lysate, value differences based on titration of 2 aliquots

**Signal of IPC3 - Protein A at background level of $H_2O$ control

*A negative control experiment for purification of AAV9 with HiTrap™ Protein A HP columns yielded no visible AAV9 elution peak in chromatography and a dPCR signal below the detection limit of background control (IPC3).*

*Selective purification of AAV9-VP3-Nb incorporating particles is possible via protein A binding.*

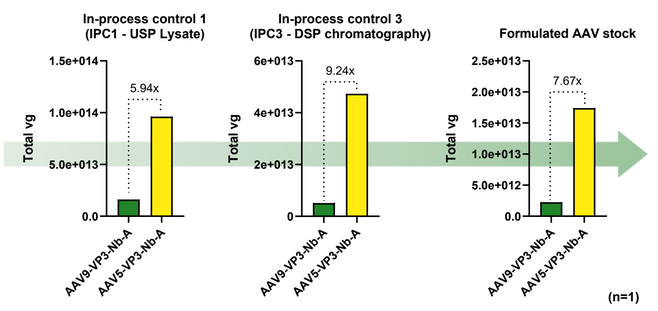
## 6 Insertion of different nanobodies into VP3 is possible




*Incorporation of a nanobody recognizing a different epitope B (VP3-Nb-B) into AAV9 is feasible. USP lysate yields are comparable to AAV9-VP3-Nb-A. Purification yields via Protein A chromatography are comparable (IPC3). Final yields are slightly lower after formulation.*

*Exchanging the nanobody to target a different cellular receptor is possible, without significantly impacting manufacturability.*

## 7 Insertion of VP3-Nbs into different AAV serotypes is possible




*Insertion of VP3-Nb-A into AAV5 shows that yields are 6 to 9-fold higher for AAV5 throughout all production steps, as expected according to our SOPs for these serotypes. The incorporation rate per AAV5 particle is comparable to AAV9 (data not shown).*

*This example confirms that incorporation of a nanobody into VP3 of another AAV serotype is feasible and our newly established manufacturing protocol is universally applicable for AAV-VP3-Nb manufacturing.*

## Summary

The novel use of VP3-incorporated nanobodies and the industrial manufacturing processes described herein demonstrate the feasibility of retargeting AAVs using nanobodies while maintaining the original VP1, VP2 and VP3 components. The presented method is a starting point for the development of an AAV-Nb platform which is modular in nature and can be utilized to further improve gene therapy safety and efficacy.

## References

[1] A.M. Eichhoff et al., Molecular Therapy Methods & Clinical Development (2019).
[2] O. Olarewaju et al., Molecular Therapy Methods & Clinical Development (2024).
[3] M.V. Hamann et al., PLoS One (2021).
[4] M.D. Hoffmann et al., NAR Molecular Medicine (2024).
[5] Patent application published as WO2025088080A1.



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