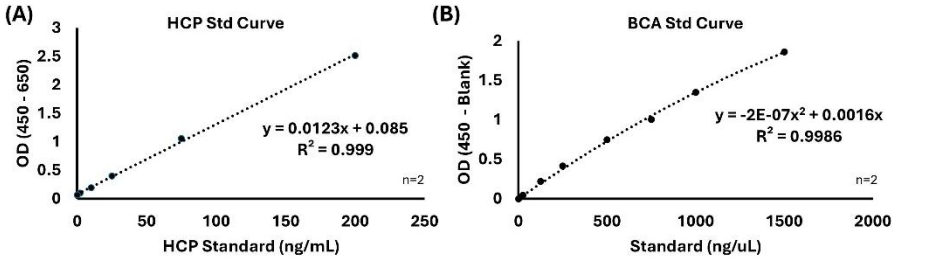
Appendix Figures/Tables:

Supplementary Figures:



Sample Name	Dilution	Total Protein (ng)	Removal (%) [a]
Before Purification	125	1.15E+06	NA
	625	1.20E+06	
Group 1	5	225.61	99.97
	25	343.50	
Group 2	5	278.46	99.97
	25	428.86	
	125	335.37	

Sample Name	Dilution	Final Conc. (ng/uL)	Total Protein (ng)	HCP (ng)	Total HCP (%) [b]
Group 1	3	62.04	62,035.35	429	0.51
Group 2	3	84.67	84,673.74	343	0.56

[a] Refer to Appendix Equation 1 for calculation of the removal percentage of host cellular protein
[b] Refer to Appendix Equation 2,3 for calculation to get host cell protein percentage. The result uses the host cell protein quantification from ELISA and Pierce™ total protein assay to get the total host cellular protein percentage

Figure S1 – Purity confirmation of rAAV from Host Cellular Contamination

Here we demonstrated the purity of rAAV after purification with Akta Avant 25 (Group 1 & 2). The purity was assessed by quantifying the amount of HCP found in the purified sample pool before and after purification. We found that less than 1% of HCP remains in the purified sample pool, which is considered insignificant.

In Figure S1, HCP contamination is quantified with HCP ELISA kits from Progen specifically for HEK293T cells and BCA protein assay kits from ThermoFisher Scientific. The limit of detection (LOD) for the HCP kit is 3.5×10^{-4} ng/µL and the LOD for the BCA kit is 20 ng/µL. ELISA based techniques are specifically targeting HCP proteins produced in the HEK293T system, and from our preliminary results, the amount of removal based on initial stock (before purification) and comparing it with the purified samples (Group 1 or 2), as shown in Equation 1:

$$HCP_{rem} = \frac{AP}{BP} * 100 \text{ [Equation 1]}$$

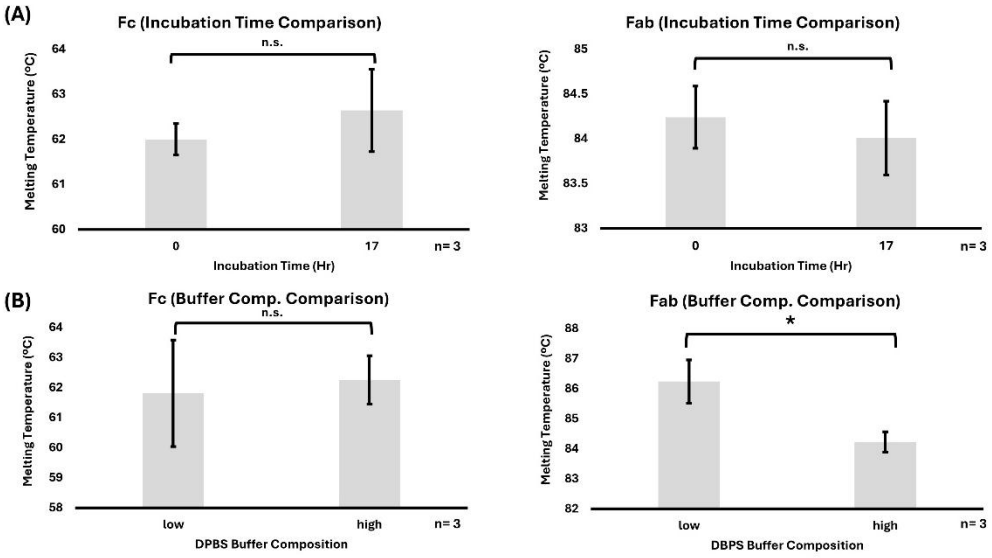
Where, HCP_{rem} is the HCP removal (%), AP is the HCP sample after purification (ng/µL), BP is the HCP sample before purification (ng/µL). A second BCA assay will be used to ensure product purity (from Figure S1 (B)), the amount of total HCP is calculated from Equation 2:

$$Total_{HCP} = \frac{(TP - AP_{tot})}{TP} * 100 \text{ [Equation 2]}$$

Where $Total_{HCP}$ is the total HCP (%), TP is the total protein based on BCA assay (ng), and AP_{tot} is the amount of HCP (ng) based on the HCP analysis from ELISA. From Equation 2, we could get the HCP_{rem}, as shown with Equation 3:

$$HCP_{rem} = 100 - Total_{HCP} \text{ [Equation 3]}$$

Based upon the preliminary data, after purification samples have less than 1% HCP contamination. Which reassures that any subsequent analysis of N-linked glycan will not have any significant interference from HCP.



Reactions [a]	mA Std [b] (µL)	10X Glyco 1 (µL)	H20 (µL)	DPBS (µL)	Incubation (Hr)	Tm 1 (°C)	Tm 2 (°C)	Summary of Condition [C]
1	12	5	30	3	17	61.23	87.03	Low DPBS
2	6		15	29	17	61.61	84.4	High DPBS, no 10X Glyco 1
3	12		35	3	17	63.35	85.64	Low DPBS, no 10X Glyco 1
4	6		17.5	26.5	17	63.16	83.84	High DPBS, no 10X Glyco 1
5	12	5	30	3	17	61.23	84.04	High DPBS
6	6			44	17	62.96	83.58	High DPBS, no 10X Glyco 1
7	12	5	30	3	0	60.84	86.04	Low DPBS
8	6	2.5	15	26.5	0	62	84.44	High DPBS
9	12	5		33	0	61.23	84.44	High DPBS
10	12			38	0	62.77	83.84	High DPBS, no 10X Glyco 1

[a] These reactions were combined and compared from reaction 1 to 10 to generate mean average with S.D. for Tm impacted by incubation and buffer comparison groups
[b] Std. Concentration: 20 mg/ml
[C] High DPBS ≥ 50% v/v

Figure S2 – Melting temperature differences observed from differential scanning fluorimetry; (A) incubation period, (B) buffer composition

From Figure S2, an assessment was conducted with monoclonal antibodies (mAb) to evaluate melting temperature changes because of a change in incubation period at 37 °C, different concentrations of DPBS, and 10X glyco buffer 1. The error bars represent conditional standard deviations (n=3). This study was used to assess the different testing conditions required for the endoglycosidase and exoglycosidase treatment. Prolonging the incubation period from 0 to 17 hours did not result in a change in melting temperature. Likewise, there was no change in melting temperature due to the addition of 10X glyco buffer 1. We observed there is a DPBS threshold (~50% v/v) that can result in a difference in melting temperature. Anything that is considered as high DPBS has ≥ 50% v/v of DPBS in the sample matrix. The difference observed could be ~2 to 3 °C and can be exacerbated for rAAV.

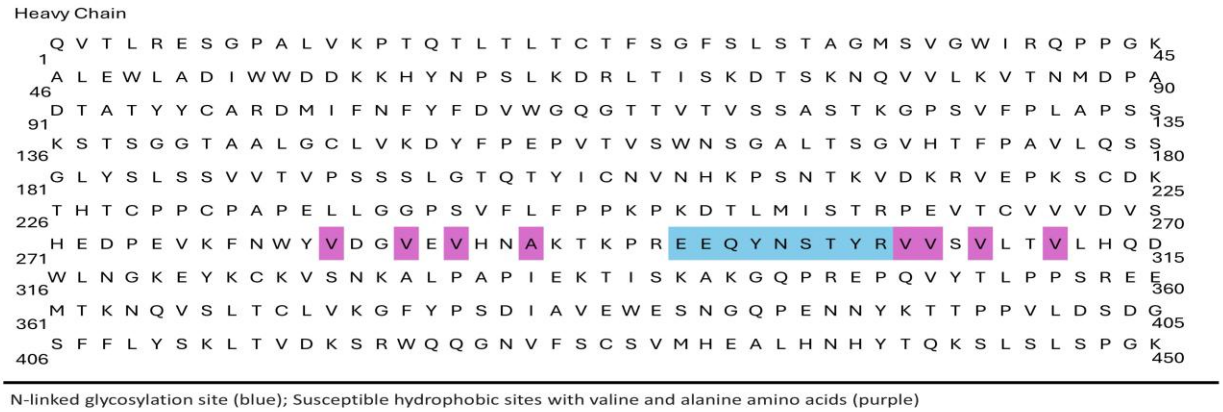


Figure S3 – NIST mAb peptide map sequence [72]

From Figure S3, the peptide mapping for NIST mAb was obtained from Mouchahoir et al. [72] for the heavy chain and used as a reference to identify potential N-linked glycan sites for mAb; where N-linked glycan typically occurs when the peptide mapping sequence is as follows: N-X-S/T, where X can be any amino acid except for proline. From literature, EEQYNSTYR site has been identified to have N-linked glycan attachment [68-72]. Hydrophobic cores are highlighted in red; these are valine (V) or alanine (A) amino acids.

Supplementary Table

Table S1. Plasmid Transient Transection Ratio Condition

Plasmid	Ratio
pAdDelta (Helper Plasmid)	2.5
AAV2/2 (8 or 9) (Rep/Cap Plasmid)	1
GFP (GOI Plasmid)	1

Total DNA used was 4 µg. 10% v/v was used as the plasmid mix cocktail volume. The cocktail volume consisted of 1:1 ratio with Plasmid DNA and PEIpro®, respectively. Opti – MEM™ media was used to dilute the cocktail mix volumes for DNA and PEIpro® prior to mixing them together and incubation at room temperature thereafter for 15 minutes.

Table S2. Adalimumab Biosimilar Reference Standard

Glycan Species	Retention Time (min.)	Theoretical mass [a]	Measured m/z	Ion	Error (ppm) [b]
G0	7.52	1577.6343	1578.4388	$[M+H]^{1+}$	509.9660
G0F	8.55	1723.6922	1724.4768	$[M+H]^{1+}$	455.2089
HM5	9.63	1495.5812	1496.3983	$[M+H]^{1+}$	546.3695
G1	10.00	1739.6871	1740.4722	$[M+H]^{1+}$	451.2881
G1F	11.10	1885.7450	1886.5084	$[M+H]^{1+}$	404.8320

G1F'	11.57	1885.7450	1886.5084	$[M+H]^{1+}$	404.8320
HM6	12.40	1658.6340	1658.4327	$[M+H]^{1+}$	-121.3649
G2F	14.27	1024.8989	1024.7827	$[M+2H]^{2+}$	-113.3800

[a] Calculated from NIST toolbox: Glyco Mass Calculator

[b] Calculated from University of Warwick toolbox: Mass Calculations: mass error and m/z from formula