YMTHE, Volume *26*

Supplemental Information

Deamidation of Amino Acids on the Surface of Adeno-Associated Virus Capsids Leads to Charge Heterogeneity and Altered Vector Function

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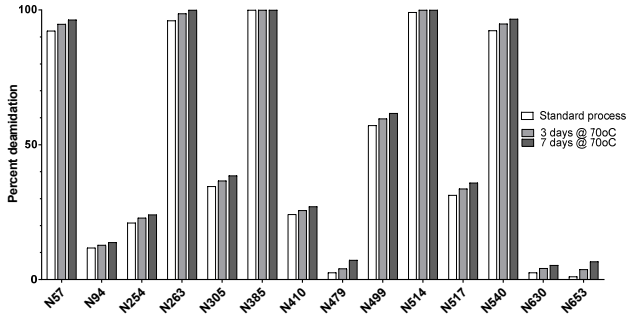
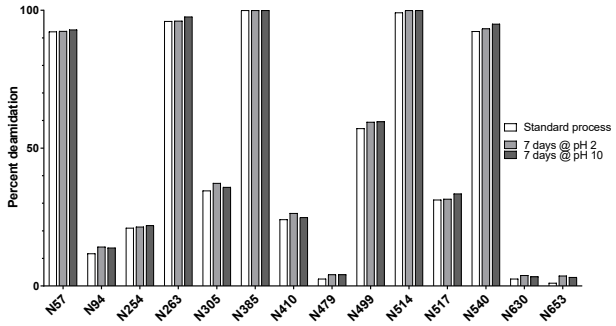
Supplemental Data

Figure S1. Determination of factors influencing AAV8 capsid deamidation

A.



B.



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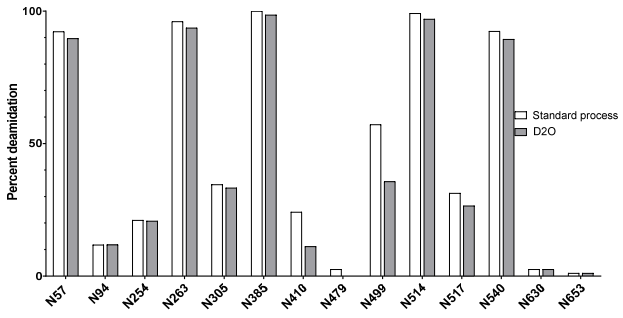

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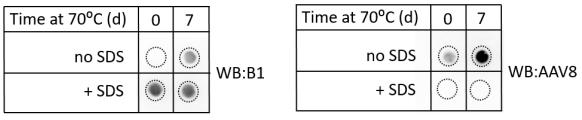
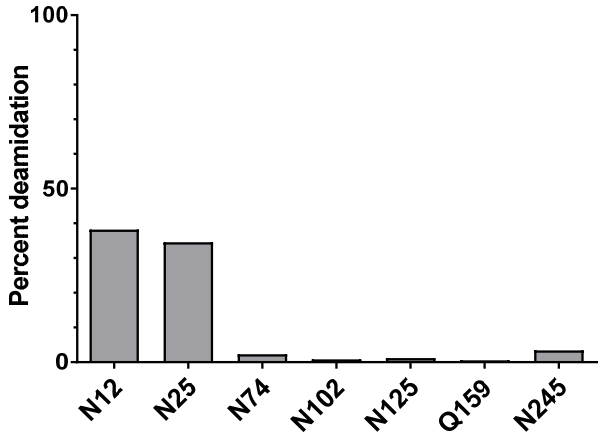


Figure S1. Determination of factors influencing AAV8 capsid deamidation. An AAV8 prep was (A) incubated at 70ºC for three or seven days, (B) exposed to pH 2 or pH 10 for seven days, or (C) prepared for mass spectrometry using D_2O in place of H_2O to determine possible sources of deamidation not intrinsic to AAV capsid formation. (D) A dot blot of vector treated as in (A) using the B1 antibody (reacts to denatured capsid) and an AAV8 conformation specific antibody (reacts to intact capsids) to assess capsid structural integrity.

Figure S2. Deamidation frequencies in non-AAV proteins

A.



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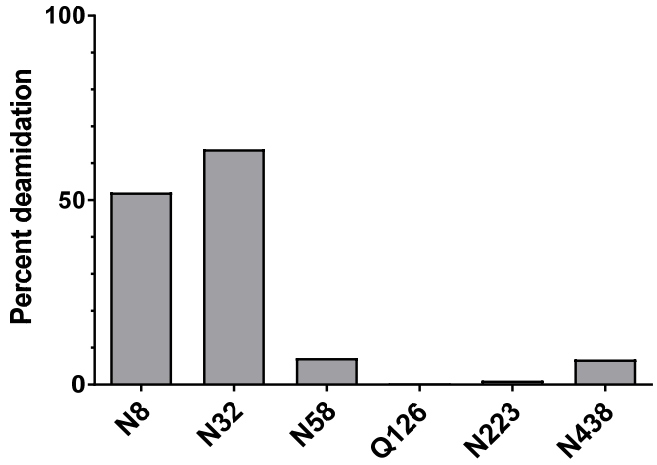


Figure S2. Deamidation frequencies in non-AAV proteins. Deamidation percentages are shown for two non-AAV recombinant proteins containing NG motifs likely to be deamidated, human carbonic anhydrase (A) and rat phenylalanine-hydroxylase (B), for comparison with AAV deamidation percentages.

Figure S3. Comparison of AAV8 percent deamidation calculated using data analysis pipelines from 2 institutions

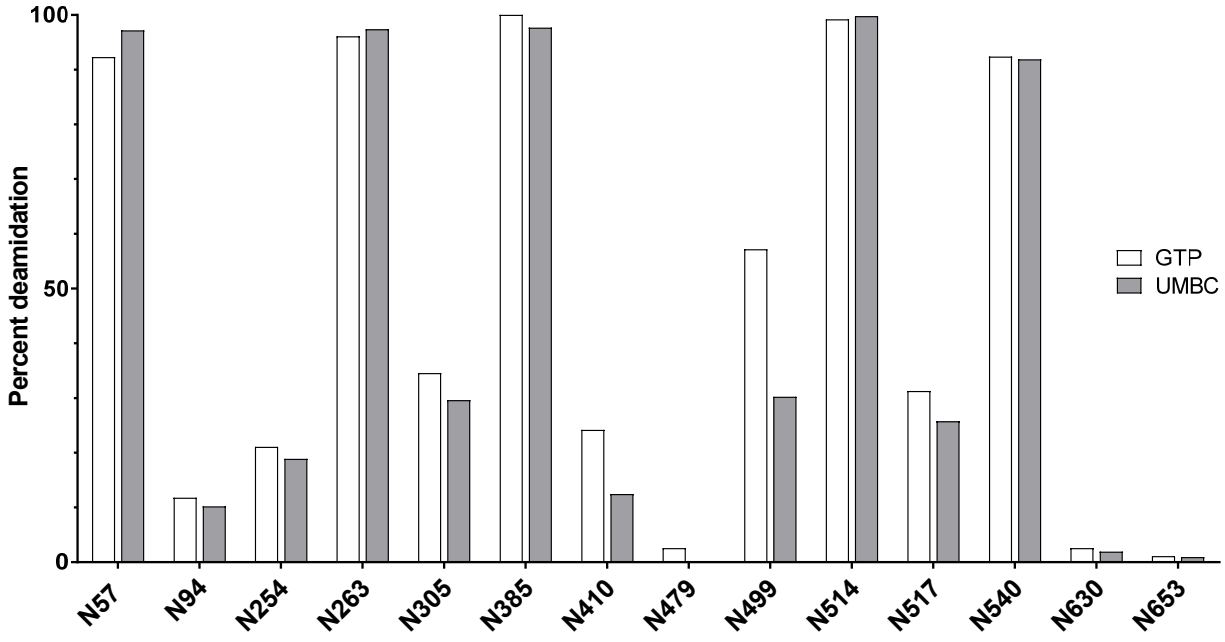


Figure S3. Comparison of AAV8 percent deamidation calculated using data analysis pipelines from two institutions. Percent deamidation at specific asparagine and glutamine residues of interest are shown for AAV8 tryptic peptides evaluated at two different institutions.

Figure S4. Analysis of asparagine and glutamine deamidation in AAV9 capsid proteins

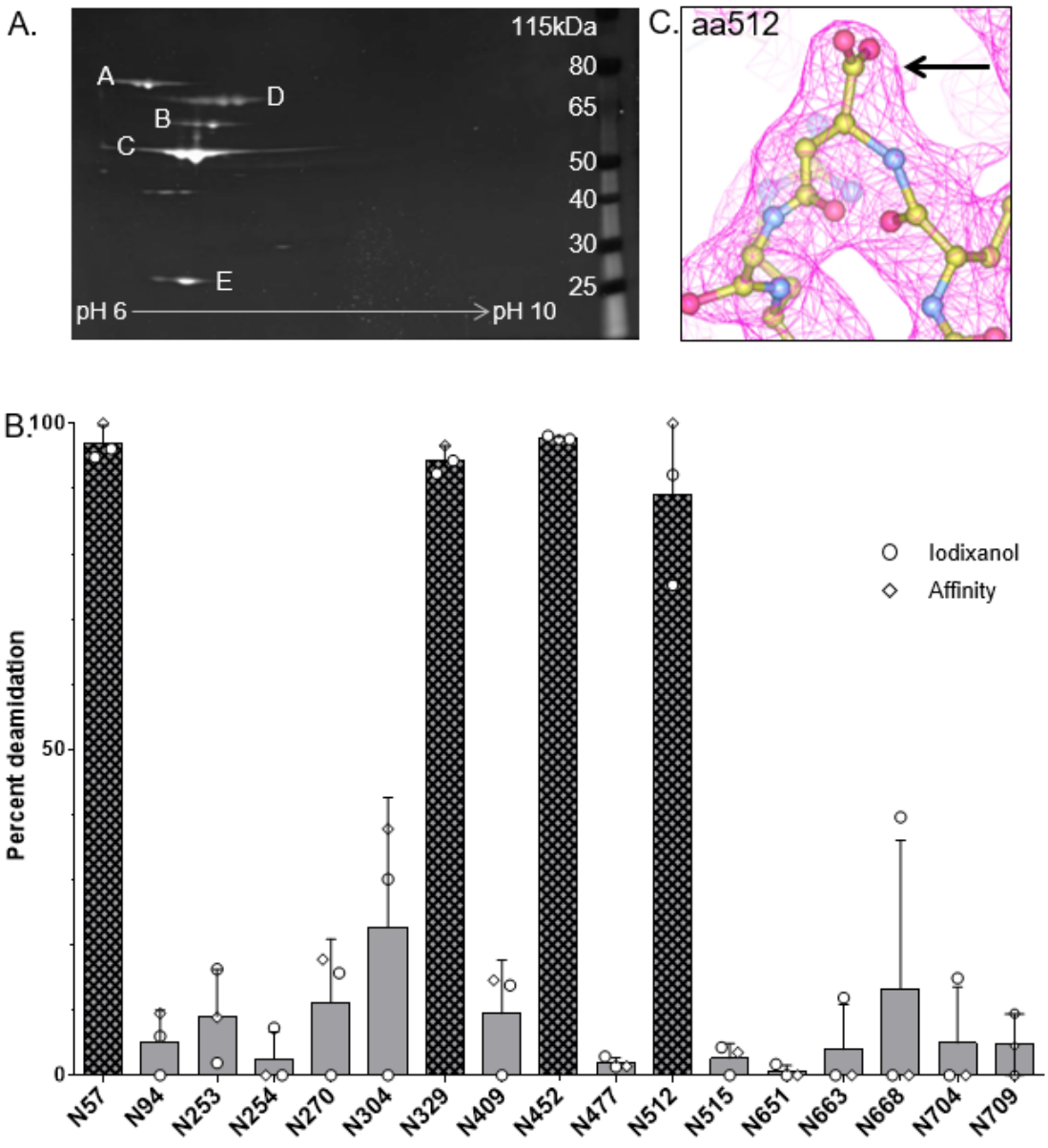
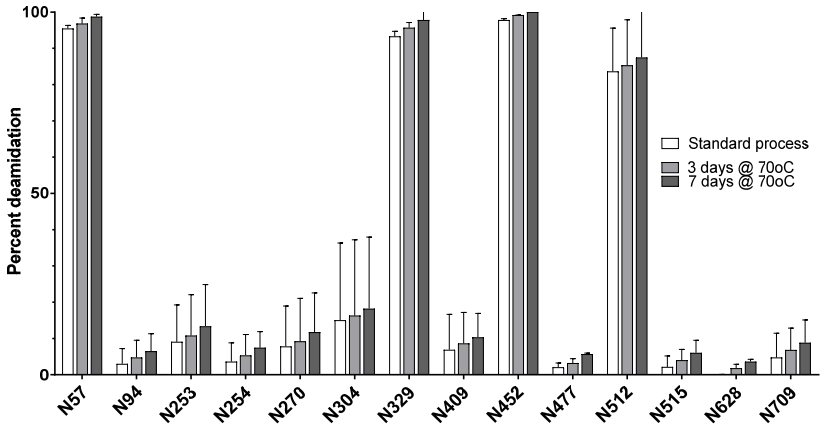


Figure S4. Analysis of asparagine and glutamine deamidation in AAV9 capsid proteins. (A) 1e11 GCs of wtAAV9 were analyzed by 2D gel electrophoresis and stained with Sypro Ruby. Protein labeling: A=VP1; B=VP2; C=VP3, D=chicken egg white conalbumin marker, E=turbonuclease marker. Isoelectric focusing was performed with a pI range of 6-10. (B) Percent deamidation at specific asparagine and glutamine residues of interest are shown for AAV9 tryptic peptides purified by different methods. Bars indicating deamidation at asparagine residues with N+1 glycines are crosshatched. Residues determined to be at least 2% deamidated in at least one prep analyzed were included. Data are represented as mean ± standard deviation. (C) Isoaspartic model of N512 is shown in the 2FoFc electron density map generated by non-biased refinement of the AAV9 crystal structure (PDB ID: 3UX1). Arrow indicates electron density corresponding to the R group of residue N512.

Figure S5. Determination of factors influencing AAV9 capsid deamidation

A.



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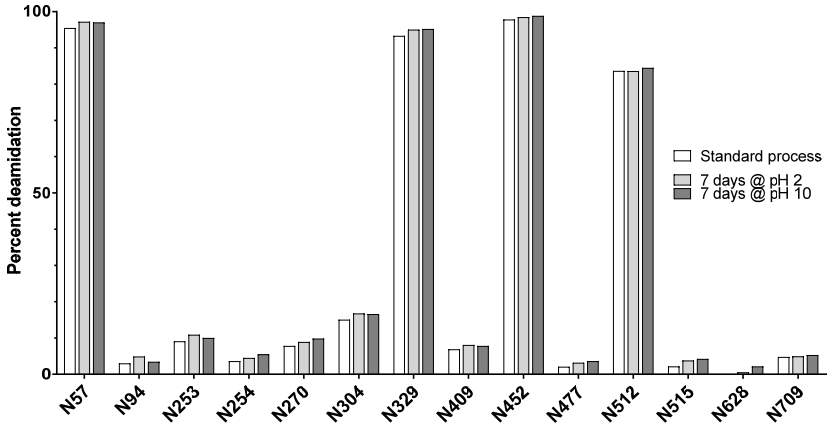

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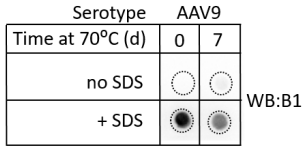
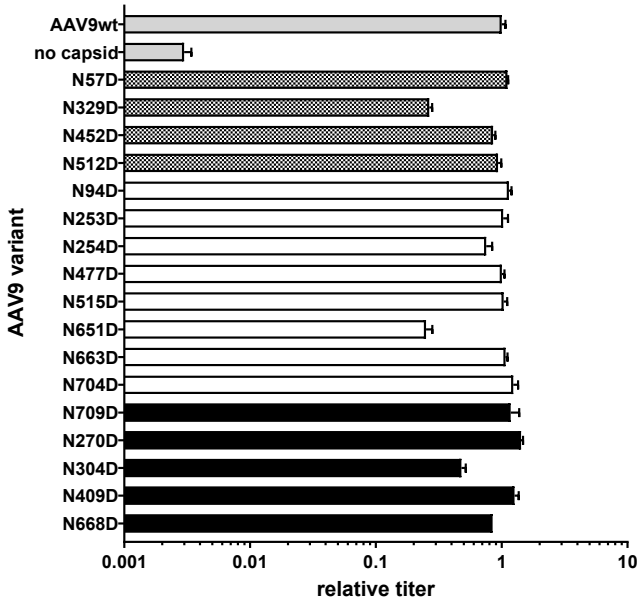


Figure S5. Determination of factors influencing AAV9 capsid deamidation. Two AAV9 preps were (A) incubated at 70°C for three or seven days or (B) exposed to pH 2 or pH 10 for seven days to determine possible sources of deamidation not intrinsic to AAV capsid formation. Data are represented as mean ± standard deviation. (C) A dot blot of vector treated as in (A) using the B1 antibody (reacts to denatured capsid) to assess capsid structural integrity.

Figure S6. The impact of genetic deamidation on AAV9 vector performance

A.



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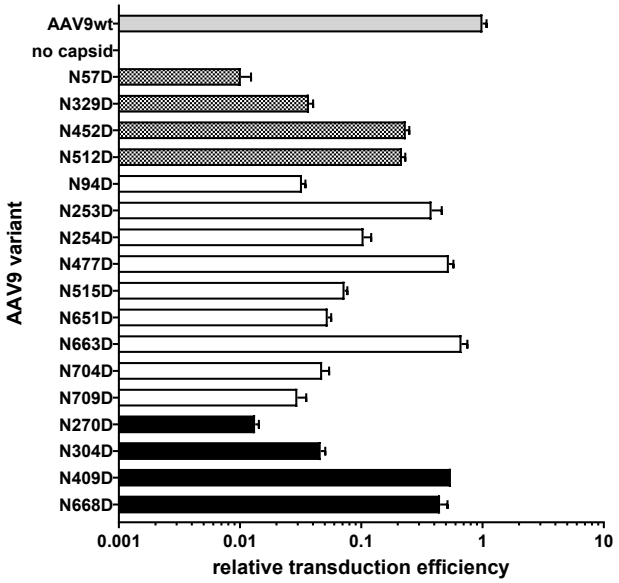
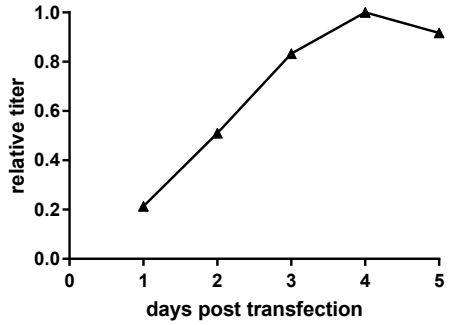


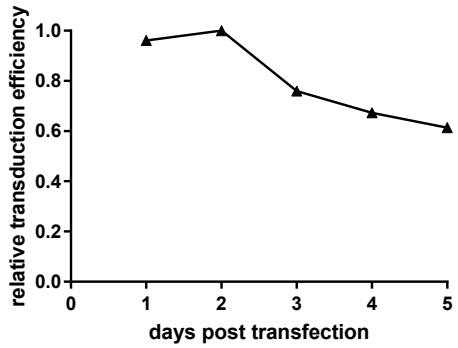
Figure S6. *In vitro* analysis of the impact of genetic deamidation on vector performance. (A) Titers of wtAAV9 and genetic deamidation mutant vectors were produced by small-scale triple transfection in 293 cells, as measured by quantitative PCR (qPCR). Titers are reported relative to the wtAAV9 control. NG sites with high deamidation (patterned bars), sites with low deamidation (white bars) and highly variable sites (black bars) are presented with wtAAV8 and a negative control. (B) The transduction efficiency of mutant AAV9 vectors producing firefly luciferase are reported relative to the wtAAV9 control. All data are represented as mean ± standard deviation.

Figure S7. AAV9 vector *in vitro* potency through time

A.



B



C.

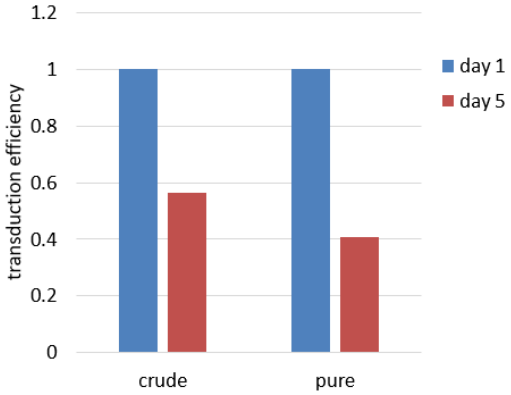


Figure S7. AAV9 vector *in vitro* potency through time. Vector production (DNAseI resistant Genome Copies, GC) for a timecourse of triple-transfected HEK 293 cells producing AAV9 vector packaging a luciferase reporter gene. GC levels are normalized to the maximum observed value. (B) Crude timecourse vector was used to transduce Huh7 cells. (C) Transduction efficiencies of vector collected 1 day post transfection vs. 5 days post transfection are shown for crude and purified vector samples. Transduction efficiency is expressed as luciferase activity/GC, normalized to the value at day 1.

Table S1. Characteristics of AAV9 deamidated residues of interest

	N+1 residue	Structural topology	Structural motif	Average % deamidation	Temperature factor (Å^2)
N57	G	N/A	N/A	97	N/A
N94	H	N/A	N/A	5	N/A
N253	N	Surface exposed	Not assigned	9	41
N254	H	Surface exposed	Not assigned	2	50
N270	D	Surface exposed	HVR I	11	65
N304	N	Buried	Alpha helix	23	35
N329	G	Surface exposed	HVR II	94	89
N409	N	Buried	Not assigned	9	36
N452	G	Surface exposed	HVR IV	98	64
N477	Y	Buried	Not assigned	2	33
N512	G	Surface exposed	HVR V	89	48
N515	S	Surface exposed	HVR V	3	47
N651	T	Buried	HI loop	1	38
N663	K	Surface exposed	HI loop	4	49
N668	S	Surface exposed	HI loop	13	52
N704	Y	Surface exposed	HVR IX	5	68
N709	N	Surface exposed	HVR IX	5	55

Table S2. Extent of deamidation observed for diverse serotypes

serotype	vector preps analyzed	Average % sequence Coverage by MS	# of NGs	average NG % deamidation	# of non NG sites observed deamidated	average non-NG % deamidation
AAV1	3	91.4	4	95.6	19	12.9
AAV3B	1	89.8	4	97.0	9	9.4
AAV4	3	84.7	4	96.2	15	15.3
AAV5	1	88.7	3	88.7	11	15.3
AAV7	1	90.9	4	92.1	9	13
AAV8	21	93.4	5	90.5	37	7.4
AAV9	7	90.2	4	95.5	26	5.3
rh32.33	1	100	3	97.4	14	16.2

Supplemental Methods

Mass spectrometry

We performed mass spectrometry analysis of samples as described in the main text. For 18O-water experiments, the capsid sample was first buffer exchanged into 100 mM ammonium bicarbonate prepared in 18O-water using Zeba spin desalting columns (Thermo Scientific, Rockford, IL). To ensure complete removal of the water in the sample, we performed the buffer exchange twice. We prepared stock solutions of 1M DTT and 1M IAM in 18O-water. We followed the same denaturation, alkylation, and digestion steps as above with 18O-water reagents and buffers.

Secondary analysis of raw mass spectrometry was performed at the University of Maryland, Baltimore County using the following method. Peaks Studio v5.3 software (Bioinformatics Solutions Inc.) was used for all mass spectrometry analysis. Data refinement of the raw data files was performed with the following parameters: a precursor m/z tolerance of ≤10ppm, and precursor charge state with a minimum of 2, maximum of 4. *De novo* sequencing of the input spectrum was performed using the Peaks algorithm with a precursor ion error tolerance of 10ppm and product ion error tolerances of 0.1Da. The digestion enzyme was set as trypsin, the variable modifications were oxidation, phosphorylation, and deamidation, and the fixed modification was carbamidomethylation of cysteine.

Primer sequences for AAV8 mutants

sequence	description
CGACAACCGGGCAAAACcagAATAGCAACTTTGCCTGG	QC mutagenic primers to change AAV8 N499 to Q
CCAGGCAAAGTTGCTATTCTGGTTTTGCCCGGTTGTCG	QC mutagenic primers to change AAV8 N499 to Q
GACAACCGGGCAAAACgacAATAGCAACTTTGCCTG	QC mutagenic primers to change AAV8 N499 to D
CAGGCAAAGTTGCTATTGTCGTTTTGCCCGGTTGTC	QC mutagenic primers to change AAV8 N499 to D
GGAGGCACGGCAcagACGCAGACTCTGGG	qc mutagenic primers to change AAV8 N459 to Q
CCCAGAGTCTGCGTCTGTGCCGTGCCTCC	qc mutagenic primers to change AAV8 N459 to Q
CAGGAGGCACGGCAgatACGCAGACTCTGG	qc mutagenic primers to change AAV8 N459 to D
CCAGAGTCTGCGTATCTGCCGTGCCTCCTG	qc mutagenic primers to change AAV8 N459 to D
ctcctcccgatgtcgcgttggagatttgc	AAV8 NA263 F
gcaaatctccaacgcgacatcgggaggag	AAV8 NA263 R
cccacggcctgactagcgttgttgagtgtta	AAV8 NA385 F
taacactcaacaacgctagtcaggccgtggg	AAV8 NA385 R

ggattagccaatgaatttcttgcattcagatggtatttggtcc	AAV8 NA514 F
ggaccaaataccatctgaatgcaagaaattcattggctaatcc	AAV8 NA514 R
tttgccaaaaatcaggatcgcgttactgggaaaaaaacg	AAV8 NA540 F
cgtttttttcccagtaacgcgatcctgatttttggcaaa	AAV8 NA540 R
ggacccttcaacgcactcgacaagggg	AAV8 NA57 F
ccccttgtcgagtgcgttgaagggtcc	AAV8 NA57 R
tggctcctcccgatgtgctgttggagatttgcttg	AAV8 NS263 F
caagcaaatctccaacagcacatcgggaggagcca	AAV8 NS263 R
cccacggcctgactactgttgttgagtgttagg	AAV8 NS385 F
cctaacactcaacaacagtagtcaggccgtggg	AAV8 NS385 R
ttagccaatgaatttctgctattcagatggtatttggtcccagcag	AAV8 NS514 F
ctgctgggaccaaataccatctgaatagcagaaattcattggctaa	AAV8 NS514 R
ttgtttgccaaaaatcaggatgctgttactgggaaaaaaacgctc	AAV8 NS540 F
gagcgtttttttcccagtaacagcatcctgatttttggcaaacaa	AAV8 NS540 R
ctccccccttgtcgaggctgttgaagggtccgag	AAV8 NS57 F
ctcggacccttcaacagcctcgacaaggggggag	AAV8 NS57 R
cagcgactcatcaacGACaactggggattccg	QC primer for AAV8 N305D
ggaggcacggcaGATacgcagactctgg	QC primer for AAV8 N459D
gacaaccgggcaaaacGACaatagcaactttgcctg	QC primer for AAV8 N499D
ccatctgaatggaagaGATtcattggctaatcctggcatc	QC primer for AAV8 N517D
cgaagcccaaagccGACcagcaaaagcagg	QC primer for AAV8 N35D
gtacctgcggtatGACcacgccgacgcc	QC primer for AAV8 N94D
gatgctgagaaccggcGACaacttccagtttacttac	QC primer for AAV8 N410D
cagactctgggcttcagcGATggtgggcctaatacaatg	QC primer for AAV8 Q467D
ccaatcaggcaaagGACtggctgccaggac	QC primer for AAV8 N479D
cacggacggcGACttccacccgtctc	QC primer for AAV8 N630D
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caggcaaagttgctattgtcgttttgcccggttgtc	QC primer for AAV8 N499D
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ggcgtcggcgtggtcataccgcaggtac	QC primer for AAV8 N94D
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gtcctggcagccagtcctttgcctgattgg	QC primer for AAV8 N479D
gagacgggtggaagtcgccgtccgtg	QC primer for AAV8 N630D
cgcaggtacaggcgtgtccttgatcaggatc	QC primer for AAV8 N653D

gcagcgactcatcaacGACaactggggattccggc	alternative longer primer to make AAV8 N305D by qc mutagenesis
GCCGGAATCCCCAGTTGTCGTTGATGAGTCGCTGC	alternative longer primer to make AAV8 N305D by qc mutagenesis
cagcgactcatcaacGACaactggggattccggc	alternative longer primer to make AAV8 N305D by qc mutagenesis
GCCGGAATCCCCAGTTGTCGTTGATGAGTCGCTG	alternative longer primer to make AAV8 N305D by qc mutagenesis
gcgactcatcaacGACaactggggattccg	alternative shorter primer to make AAV8 N305D by qc mutagenesis
CGGAATCCCCAGTTGTCGTTGATGAGTCGC	alternative shorter primer to make AAV8 N305D by qc mutagenesis
ctctgggcttcagcGAAggtgggcctaatac	mutagenic QC primer to make aav8 Q467E
GTATTAGGCCCACCTTCGCTGAAGCCCAGAG	mutagenic QC primer to make aav8 Q467E
cctcggacccttcGACggactcgacaagg	QC primer for AAV8 N57D
tacaagcaaatctccGACgggacatcgggaggag	QC primer for AAV8 N263D
ctacctaacactcaacGACggtagtcaggccgtg	QC primer for AAV8 N385D
ctgggaccaaataccatctgGATggaagaaattcattggctaatc	QC primer for AAV8 N514D
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