Supplementary Materials for

SEROTYPE-DEPENDENT PACKAGING OF LARGE GENES IN ADENO-ASSOCIATED VIRAL VECTORS RESULTS IN EFFECTIVE IN VIVO GENE DELIVERY

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The PDF includes: Methods, Table S1, Table S2, Table S3, Table S4, Fig. S1 and Probe sequences.

METHODS:

Statistical analysis

The purpose of one-way ANOVA is to find out whether data from several groups have a common mean. That is, to determine whether the groups are actually different in the measured characteristic. One-way ANOVA is a simple special case of linear model. The standard ANOVA table has columns for the sums of squares, degrees of freedom, mean squares (SS/df), F statistic, and p-value. The F statistic is used to do a hypothesis test for finding out the effect of the main factor (grouping variable) on the mean of the observed data. (This test compares the variance explained by the main factor to the left over variance that cannot be explained). Sometimes it may be needed to determine not just whether there are any differences among the means, but specifically which pairs of means are significantly different. If this is the case, ad-hoc procedures known as multiple comparison procedures are designed to compensate for multiple tests.

For the purpose of the statistical analysis we considered 8.9 kb genome and ≤ 4.7 kb genome, separately. For each genome we divided our data into eight groups having "rAAV serotype" as category of defining characteristics. In this framework we performed a one-way analysis of variance (ANOVA) for testing the effects of the rAAV serotype factor (grouping variable) on the mean of the observed response (titers/yield).

1) 8.9 kb genome

Table S2 summarizes the results of our analysis. The p-value of the rAAV serotype effect is 9.7096e-005. This is a strong indication that the measurement vary strongly from a rAAV serotype to another. In order to determine which pairs of rAAV serotype levels are significantly different, and which are not, we performed a posterior multiple comparison test at level 0.05, with a Bonferroni adjustment for multiplicity. The main result is depicted in Fig. S1A. As can be seen the six groups rAAV2/2, 2/3, 2/4, 2/7, 2/8 and 2/9 (red bars) have population marginal means significantly different from the 2/5 group (blue bar).

2) ≤ 4.7 kb genome

Table S3 summarizes the results of our analysis. The p-value of 0.277 indicates that in this case the effect of rAAV serotype is not significant. Given that there is no evidence of difference between different rAAV serotypes for 8.9 kb genome, we computed the comparison intervals for the titer means, following the same procedure as for the 8.9 kb genome, and we depicted the results in Fig. S1B. As you can see the eight groups 2/1, 2/2, 2/3, 2/4, 2/5, 2/7, 2/8 and 2/9 (blue bars) population marginal means are not significantly different.

Multiple comparison procedure explanation

In a one-way analysis of variance, you compare the means of several groups to test the hypothesis that they are all the same, against the general alternative that they are not all the same. Sometimes this alternative may be too general. You may need information about which pairs of means are significantly different, and which are not. A test that can provide such information is called a "multiple comparison procedure."

When you perform a simple t-test of one group mean against another, you specify a significance level that determines the cutoff value of the t statistic. For example, you can

specify the value alpha = 0.05 to insure that when there is no real difference, you will incorrectly find a significant difference no more than 5% of the time. When there are many group means, there are also many pairs to compare. If you applied an ordinary t-test in this situation, the alpha value would apply to each comparison, so the chance of incorrectly finding a significant difference would increase with the number of comparisons. Multiple comparison procedures are designed to provide an upper bound on the probability that any comparison will be incorrectly found significant.
Table S4 contains the results of the test in the form of a five-column matrix. Each row of the matrix represents one test, and there is one row for each pair of groups. The entries in the row indicate the means being compared, the estimated difference in means, and a confidence interval for the difference. The critical value vas computed using the critical value from the t distribution, after a Bonferroni adjustment to compensate for multiple comparisons. For example, row 11 contains the following entries:

2	5	-613460608509,8289	-327166666666,6666	-40872724823,504395

These numbers indicate that the mean of group rAAV2/2 minus the mean of group rAAV2/5 is estimated to be -327166666666,6666, and a 95% confidence interval for the true mean is [-613460608509,8289 -40872724823,504395]. In this example the confidence interval does not contain 0.0, so the difference is significant at the 0.05 level. If the confidence interval did contain 0.0, the difference would not be significant at the 0.05 level. The results from Table S4 are summarized in Fig. S1. This figure shows the comparisons intervals around each group mean (1). Note: Intervals can be used for testing but are not simultaneous confidence intervals.
As a general rule in this case, two means are significantly different if their intervals are disjoint, and are not significantly different if their intervals overlap. What we can conclude then from Table S4 and Fig. S1 is that the six groups rAAV2/2, 2/3, 2/4, 2/7, 2/8 and 2/9 (red bars) have population marginal means significantly different from the 2/5 group at 0.05 level.

1. Tamhane, A.C., Hochberg, Y., and Dunnett, C.W. 1996. Multiple test procedures for dose finding. *Biometrics* 52:21-37.

Table S1. Titers (average ± standard error) of rAAV2/1, 2, 3, 4, 5, 7, 8, 9 containing genomes of 8.9 kb or ≤4.7 kb.

	8.9 kb	**≤4.7 kb**	**8.9kb /≤4.7 kb**
rAAV2/1	1.50e+11 (n=3)	3.90e+12 (n=23)	0.038
rAAV2/2	4.00e+10 (n=3)	2.00e+12 (n=7)	0.020
rAAV2/3	3.50e+10 (n=4)	7.60e+12 (n=3)	0.005
rAAV2/4	1.90e+10 (n=4)	2.10e+12 (n=3)	0.009
rAAV2/5	3.70e+11 (n=12)	5.40e+12 (n=20)	0.069
rAAV2/7	5.10e+10 (n=3)	4.30e+12 (n=7)	0.012
rAAV2/8	5.00e+10 (n=3)	4.10e+12 (n=26)	0.012
rAAV2/9	7.90e+10 (n=3)	5.50e+12 (n=12)	0.014

N.B.: 8.9 kb/ ≤4.7 kb: ratio between titers of rAAV serotypes with genome size of 8.9 kb and the same rAAV serotypes with genome size of ≤4.7 kb. n: number of rAAV preparations.

Table S2. Anova for 8.9 kb genome: Analysis of variance.

Source	**Sum Sq**	**d.f.**	**Mean Sq**	**F**	**Prob>F**
Experiment	7.90065e+023	7	1.12866e+023	6.89	9.70959e-005
Error	4.423e+023	27	1.63815e+022		
Total	1.23237e+024	34			

Table S3. Anova for ≤ 4.7 kb genome: Analysis of variance.

Source	**Sum Sq**	**d.f.**	**Mean Sq**	**F**	**Prob>F**
Experiment	1.39387e+026	7	1.99124e+025	1.26	0.277
Error	1.46552e+027	93	1.57582e+025		
Total	1.6049e+027	100			

Table S4. ANOVA posthoc analysis for large genomes.

Group label	Group label	0.05CIntervalA	Difference in Means	0.05CIntervalB
rAAV2/1	rAAV2/2	-250969707946.204000	111166666666.666000	473303041279.537000
rAAV2/1	rAAV2/3	-222855893605.779000	115891666666.666000	454639226939.112000
rAAV2/1	rAAV2/4	-207030893605.779000	131716666666.666000	470464226939.112000
rAAV2/1	rAAV2/5	-502293941843.162000	-216000000000.000000	70293941843.162100
rAAV2/1	rAAV2/7	-262469707946.204000	99666666666.666500	461803041279.537000
rAAV2/1	rAAV2/8	-261136374612.870000	100999999999.999000	463136374612.870000
rAAV2/1	rAAV2/9	-290469707946.204000	71666666666.666500	433803041279.537000
rAAV2/2	rAAV2/3	-334022560272.446000	4724999999.999920	343472560272.445000
rAAV2/2	rAAV2/4	-318197560272.446000	20549999999.999900	359297560272.445000
rAAV2/2	rAAV2/5	-613460608509.828000	-327166666666.666000	-40872724823.504300
rAAV2/2	rAAV2/7	-373636374612.870000	-11500000000.000000	350636374612.870000
rAAV2/2	rAAV2/8	-372303041279.537000	-10166666666.666600	351969707946.204000
rAAV2/2	rAAV2/9	-401636374612.870000	-39500000000.000000	322636374612.870000
rAAV2/3	rAAV2/4	-297794300049.144000	15825000000.000000	329444300049.144000
rAAV2/3	rAAV2/5	-587960752869.739000	-331891666666.666000	-75822580463.593600
rAAV2/3	rAAV2/7	-354972560272.445000	-16224999999.999900	322522560272.445000
rAAV2/3	rAAV2/8	-353639226939.112000	-14891666666.666500	323855893605.779000
rAAV2/3	rAAV2/9	-382972560272.445000	-44224999999.999900	294522560272.445000
rAAV2/4	rAAV2/5	-603785752869.739000	-347716666666.666000	-91647580463.593600
rAAV2/4	rAAV2/7	-370797560272.445000	-32049999999.999900	306697560272.445000
rAAV2/4	rAAV2/8	-369464226939.112000	-30716666666.666500	308030893605.779000
rAAV2/4	rAAV2/9	-398797560272.445000	-60049999999.999900	278697560272.445000
rAAV2/5	rAAV2/7	29372724823.504400	315666666666.666000	601960608509.828000
rAAV2/5	rAAV2/8	30706058156.837700	317000000000.000000	603293941843.162000
rAAV2/5	rAAV2/9	1372724823.504450	287666666666.666000	573960608509.828000
rAAV2/6	rAAV2/8	-360803041279.537000	1333333333.333320	363469707946.204000
rAAV2/7	rAAV2/9	-390136374612.870000	-28000000000.000000	334136374612.870000
rAAV2/8	rAAV2/9	-391469707946.204000	-29333333333.333300	332803041279.537000

N.B.: statistically significant differences are depicted in yellow.

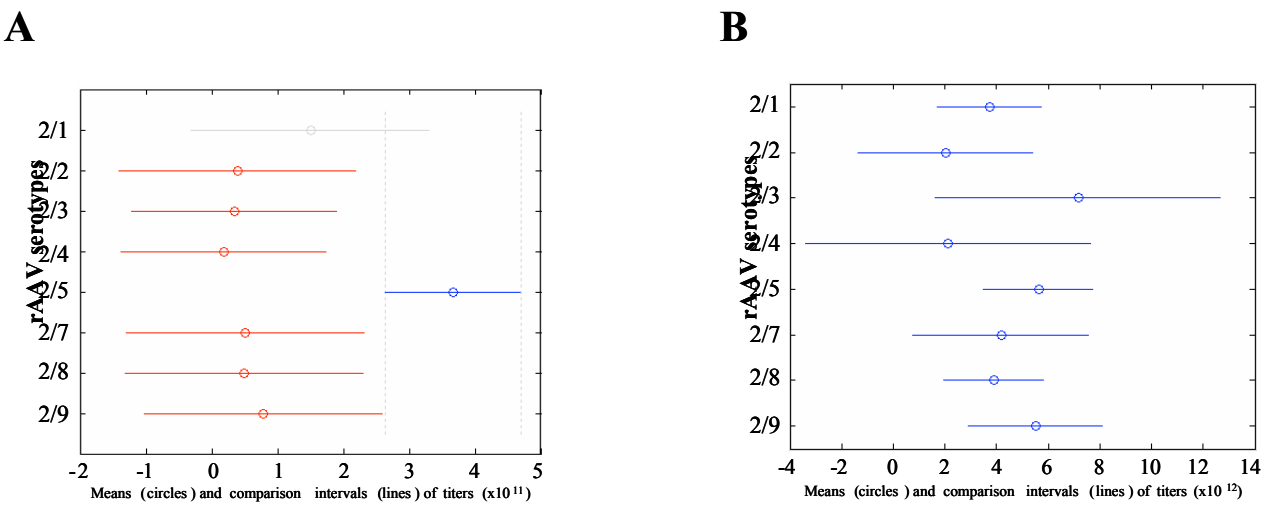

Fig. S1. Statistical analyses of titers from rAAV vectors packaging ≤ 4.7 kb vs 8.9 kb genomes. (A) Mean comparison intervals of titers of rAAV2/1, 2, 3, 4, 5, 7, 8, 9 containing *Abca4* (rAAV genome size: 8.9 kb) obtained by the multiple comparisons test procedure after the one-way Anova. The rAAV highlighted in blue (rAAV2/5) has a titer mean that is significantly different from the means of those highlighted in red. **(B)** Mean comparison intervals of titers of rAAV2/1, 2, 3, 4, 5, 7, 8, 9 containing genome size of ≤4.7 kb obtained by the multiple comparisons test procedure. The titers of the various serotypes do not differ significantly.

Probe sequences used for Southern blot analyses:

Poly A probe (221 bp)

gatctgcctcgactgtgccttctagttgccagccatctgttgtttgcccctcccccgtgccttccttgaccctggaaggtgccactcc
cactgtcctttcctaataaaatgaggaaattgcatcgcattgtctgagtaggtgtcattctattctgggggggtggggtggggcagga
cagcaaggggaggattgggaagacaatagcaggcatgctggggac

Probe 2 (2025 bp)

ataccaccatgtcccagaggcaaagctggtggaatgcattggtcaagaacttatcttcctccttccaaacaagaatttcaagcaga
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