A Multiplexed Epitope-Tagging Platform for Parallel In Vivo Assessment of AAV Capsid Variants

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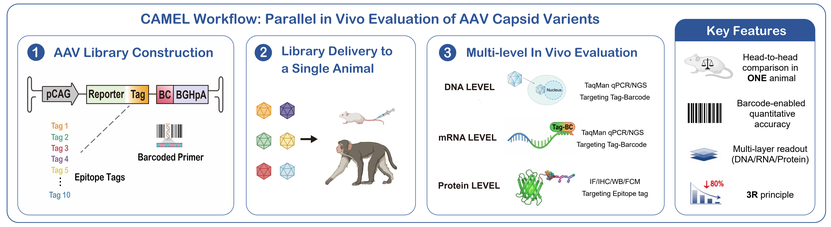
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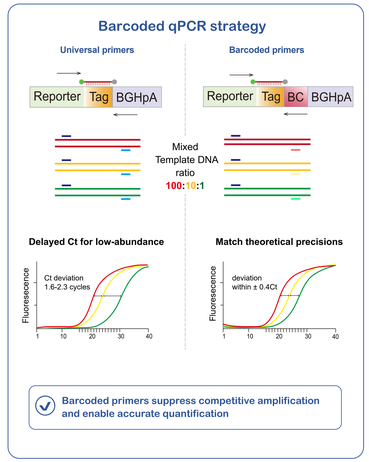
INTRODUCTION

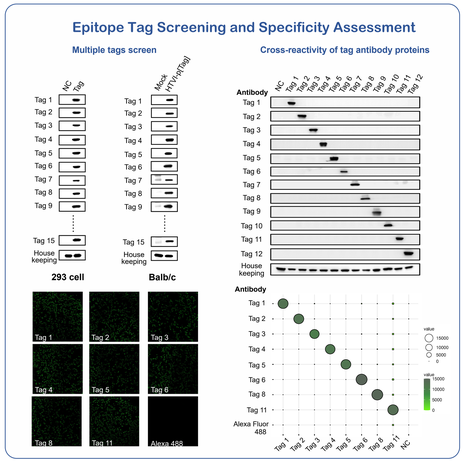
Efficient in vivo evaluation of AAV capsid variants remains a major hurdle in vector engineering. Although high-throughput libraries can now be generated rapidly, their functional validation still depends on low-throughput methods, where each variant is tested in an individual animal. This approach limits scalability and introduces significant inter-animal variability, hindering direct head-to-head comparisons. While existing multiplexing strategies improve throughput via pooled analysis, they typically rely on NGS-based DNA or RNA quantification and lack the spatial resolution or protein-level readouts necessary for comprehensive tissue tropism profiling.

To address these limitations, we developed CAMEL (Capsid Assessment via a Multiplexed Epitope-tagging Library), a platform for the parallel, multi-layer evaluation of AAV capsids within a single subject. By coupling unique epitope tags with corresponding barcoded PCR primers, CAMEL enables the high-fidelity, multi-layer quantification of vector genome biodistribution, transcriptional activity , and functional protein expression. By facilitating head-to-head comparisons within the same animal, CAMEL eliminates inter-animal variability and provides a robust framework for direct performance ranking. This scalable approach streamlines the identification of superior AAV variants for therapeutic applications.

METHODS



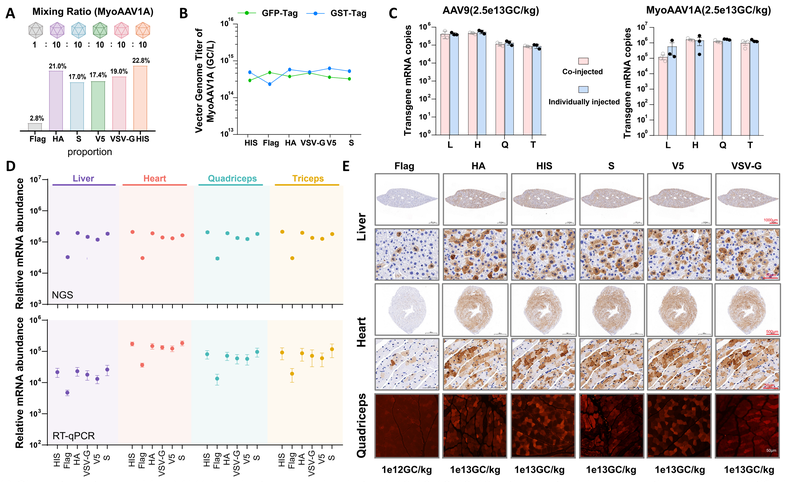




Fifteen candidate epitope tags were screened by WB and IF to evaluate detection sensitivity and spatial expression, and further validated in vivo via hydrodynamic tail vein injection. Cross-reactivity was assessed by WB and antibody matrix analysis; representative Western blot images and bubble plots demonstrate the high specificity of each tag–antibody pair. Cross-reactive candidates were excluded.

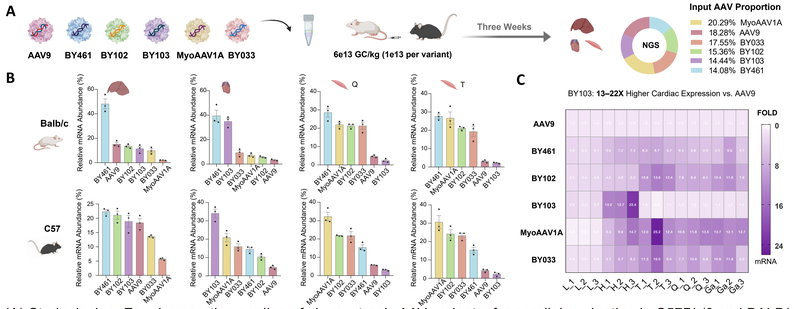
RESULTS

Figure 1. Validation of the CAMEL Platform for High-Fidelity In Vivo Quantitative Screening



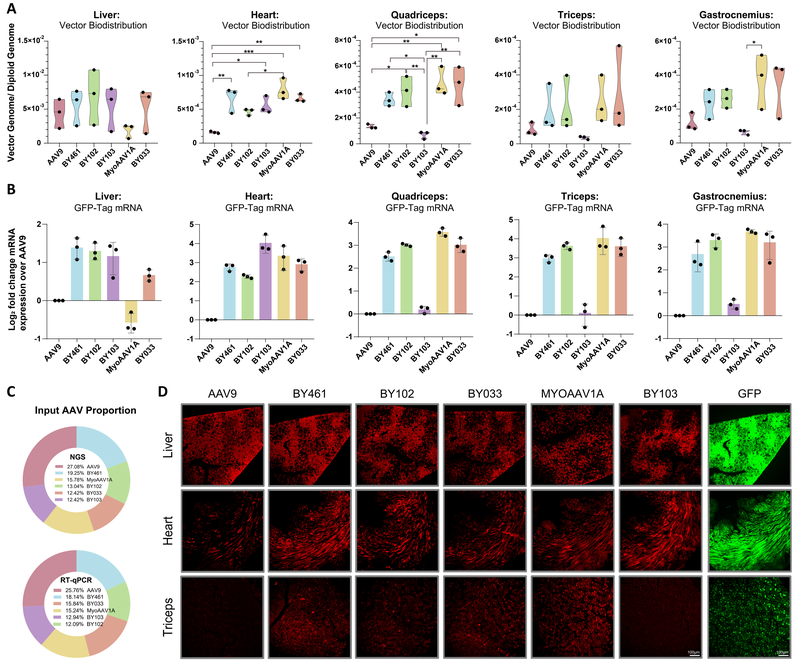
(A) Six epitope-tagged MyoAAV1A variants were mixed at a 1:10:10:10:10:10 ratio; post-mixing proportions were quantified by NGS. (B) Vector packaging titers across reporter-tag combinations, indicating minimal impact of tags on packaging efficiency. (C) Transgene mRNA expression (qPCR copy number) was compared between individual and pooled injections across multiple tissues (Liver, Heart, Quadriceps, Triceps). No significant differences or cross-interference were observed, validating that variants behave identically whether administered alone or in a complex mixture. (D) In vivo mRNA levels after CAMEL co-injection measured by NGS (top) and qPCR (bottom). (E) Representative IHC and IF images. Data are presented as Mean ± SEM (n=3).

Figure 2. Multi-strain mRNA Expression Analysis Reveals Robust and Divergent Muscle Targeting



(A) Study design. Equal-proportion pooling of six myotropic AAV variants for parallel evaluation in C57BL/6 and BALB/c mice. (B) Relative contribution of each variant to total transgene mRNA within multiple tissues, quantified by RT-qPCR. Results reveal strain-dependent tropism for BY461 and identify BY103 as a superior heart-targeting variant across both strains. (C) Heatmap showing fold-changes in mRNA expression (from B) relative to AAV9 in C57BL/6 mice. L: Liver; H: Heart; Q: Quadriceps; T: Triceps; Ga: Gastrocnemius. Data are presented as Mean ± SEM (n=3).

Figure 3. Multi-level Analysis of Myotropic Candidates Reveals Divergent Tissue Tropism



(A) Quantification of vector genome copies per diploid cell distinct tissue-specific enrichment patterns among variants, which significantly deviate from the AAV9 reference profile. (B) Analysis of mRNA expression (log_2 fold change relative to AAV9) highlights functional divergence of candidates: BY103 exhibits selective and significant enhancement in cardiac tissue, whereas the other four variants show concurrent enrichment in both cardiac and skeletal muscle tissues. (C) Donut charts illustrate the relative proportions of six AAV variants in the pooled library as determined by NGS (top) and barcoded RT-qPCR (bottom). The highly concordant distributions between the two methods confirm the robustness and quantitative reliability of the CAMEL platform. (D) Immunofluorescence detection of transgene proteins using an Alexa Fluor 647 secondary antibody against specific epitope tags in the liver, heart, and triceps. The green channel serves as the GFP reference. Scale bars = 100 µm. Statistical analysis: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ via one-way ANOVA with Tukey's post-test. Data are presented as Mean ± SEM.

CONCLUSIONS

>> The CAMEL platform, integrated with a barcode-specific qPCR strategy, effectively eliminates universal primer bias. Ensures high quantitative fidelity across DNA, mRNA, and functional protein levels.

>> By enabling parallel evaluation of multiple variants within a single animal, the platform achieves an 80% reduction in animal usage without compromising the accuracy of head-to-head capsid comparisons.

>> Identified BY103 as a potent heart-tropic AAV capsid variant and revealed strain-dependent differences in the performance of BY461, highlighting the need for multi-strain profiling in AAV engineering.