Identification of Novel Tissue-tropic AAV Capsids Using an In Vivo-Calibrated Barcode Library

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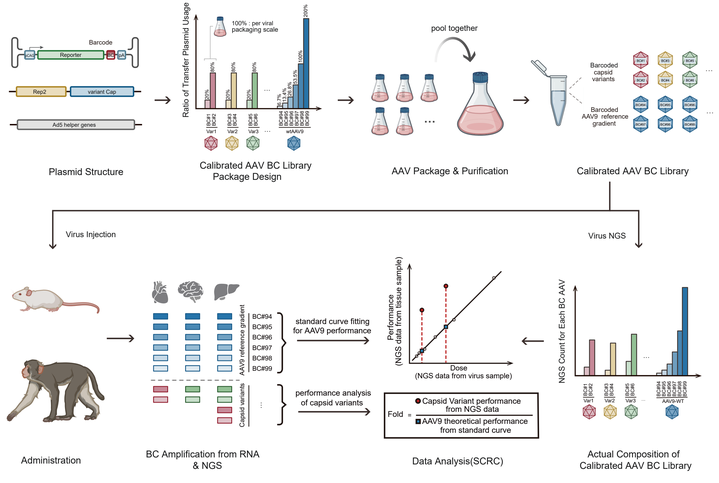
INTRODUCTION

The discovery of novel AAV capsids has been significantly accelerated by barcoded library screening. Barcoded library screening enables parallel assessment of AAV variants via NGS. However, traditional methods require laborious individual packaging and titration of each variant before equimolar pooling. Despite meticulous execution, this process inevitably introduces substantial compositional bias, which intensifies with library complexity. These titer discrepancies preclude performance comparisons at equivalent doses, thereby preventing accurate variant ranking.

To address these limitations, we developed **CALIBRATOR** (Calibrated AAV Libraries with Internal Barcode-Referenced Analytes for True Output Ranking). CALIBRATOR establishes an in vivo calibration system by co-delivering a gradient of internal reference standards. Together with the **SCRC** (Standard-curve-based Regression Calibration) method, which constructs an independent standard curve for each sample using the internal reference gradient, this approach enables precise quantitative calibration by mapping raw NGS data onto regression models. This system effectively transforms relative NGS read counts into absolute quantitative metrics, ensuring the fidelity of high-throughput capsid discovery.

METHODS

The CALIBRATOR Platform for Precision AAV Discovery

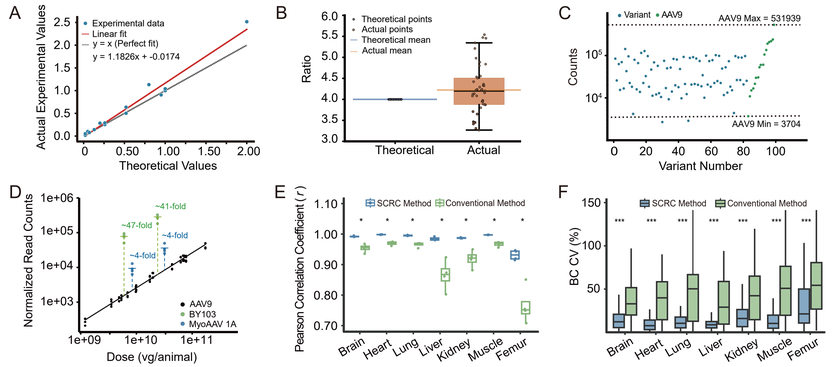


Barcode sequences are inserted into the 3′ UTR of the transgene cassette during plasmid construction. During packaging, each capsid variant is assigned two unique barcodes (at a 4:1 ratio), while the AAV9-based reference gradient is established by pooling AAV9 plasmids at pre-defined ratios with distinct barcodes. All components are individually packaged and then pooled for unified purification, ensuring a homogeneous input library with an integrated in vivo dose benchmark. Following systemic administration, tissue RNA is extracted for NGS analysis. Finally, the SCRC algorithm fits sample-specific regression models based on the internal AAV9 gradient to convert raw sequencing counts into normalized dose-equivalents. This determines the fold change of each variant relative to the AAV9 dose-response curve, enabling robust ranking across diverse tissues and species.
CALIBRATOR: Calibrated AAV Libraries with Internal Barcode-Referenced Analytes for True Output Ranking; SCRC: Standard-curve-based Regression Calibration.

RESULTS

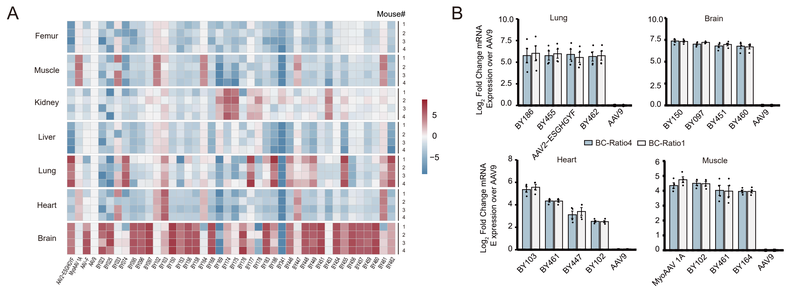
CALIBRATOR Demonstrates High Quantitative Accuracy and Algorithmic Superiority

BALB/c mice (n = 4) were systemically administered 2 × 10 ¹² vg of a calibrated library to validate SCRC performance at the transcriptional level.



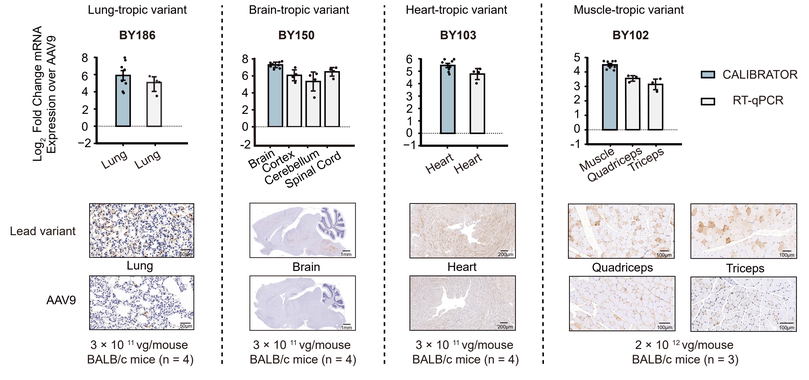
(A–C) Library Construction and Quality Control. (A) Post-packaging analysis confirmed that the experimental AAV9 gradient strongly correlated with theoretical values (R^2 > 0.95), ensuring linearity. **(B)** The dual-barcode ratio remained highly consistent (median = 4.138) across the library, indicating minimal systematic bias. **(C)** Analysis of library distribution showed that 97.56% of variant data points fell within the AAV9 reference range, ensuring that the internal benchmark provides sufficient dynamic range for accurate variant ranking.
(D) In-Tissue mRNA Calibration. SCRC established robust standard curves (R^2 > 0.9 in heart) across all evaluated organs based on tissue-specific mRNA transcripts. Representative data from the heart demonstrates precise fold-change quantification for variants such as BY103 and MyoAAV 1A relative to the internal AAV9 dose-response curve. **(E–F) Algorithmic Benchmarking.** Compared to the conventional method (defined as point-to-point matching based on a single-dose reference), SCRC significantly improved internal consistency of mRNA quantification, yielding higher Pearson correlation coefficients (r) between dual barcodes (E) and substantially lower coefficients of variation (CV) across individual animals (F). ***p<0.001, *p<0.05.

In Vivo Profiling and Identification of Tissue-Specific AAV Variants



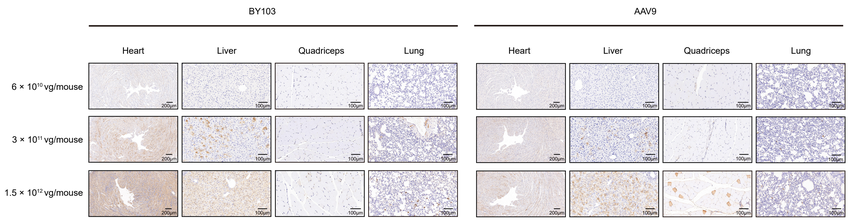
(A) Landscape of Tissue-Specific mRNA Expression. The heatmap shows relative mRNA expression levels (Log_2 fold change over AAV9) of 41 capsid variants. Variants were profiled across seven key tissues in mice (n = 4). This comprehensive profiling identified candidates with distinct tropism shifts. **(B) Rank-Ordering of Lead Variants by Transduction Potency.** Top-performing candidates were ranked by their geometric mean fold-change in mRNA expression. These data validated the superior performance of lead variants relative to AAV9.

Individual Orthogonal Validation of Lead Candidates



Individual RT-qPCR results **(upper panel)** across diverse tissues correlated strongly with SCRC-calibrated fold-change metrics, confirming the platform's reliability in prioritizing potent capsids. Representative IHC staining **(lower panel)** further corroborated the superior transduction efficiency of these leads relative to AAV9. These orthogonal findings demonstrated that absolute quantification derived from CALIBRATOR reliably translated to single-variant performance, ensuring high-fidelity discovery from mRNA-based library to individual leads.

Characterization of the Cardiotropic Variant BY103



Representative IHC images for variant BY103 **(left)** and parental AAV9 **(right)** across a three-dose gradient. BY103 demonstrated a significant enhancement in cardiac transduction efficiency, reaching ~45% IHC-positive area at the dose (3 × 10 ¹¹ vg/mouse). While BY103 showed high potency in both the heart and liver, it exhibited a markedly improved heart-to-liver selectivity ratio compared to AAV9. Notably, BY103 maintained minimal transduction in the quadriceps, distinguishing it from other heart-tropic variants that often show collateral affinity for skeletal muscle.

CONCLUSION

>> The CALIBRATOR platform is a powerful screening system that achieves absolute library quantification with high-fidelity accuracy to ensure the reliable translation of high-throughput rankings into validated leads.

>> BY103 is a high-potency variant that achieves superior cardiac selectivity with a significantly improved heart-to-liver ratio and robust de-targeting of skeletal muscle.