An Automated, High-Throughput Viral mRNA Capture Platform for In Vivo AAV Capsid-Directed Evolution

Yangyang Zhong, Qingyu Deng, Ruixue Chen, Yan Jiao, Ying Bai, Gang Wang

Byongen Therapeutics, Beijing, China Correspondence: gang.wang@byongen.com

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INTRODUCTION

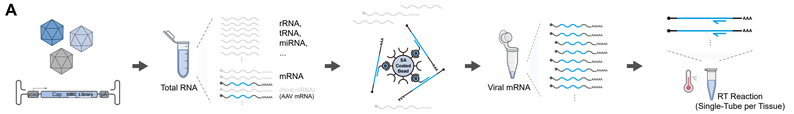
Directed evolution of Adeno-associated virus (AAV) capsids relies on accurate in vivo quantification of variant performance. Viral mRNA–based readouts provide a direct measure of productive transduction, but their application is fundamentally hindered by sparse transcript abundance and overwhelming host RNA background, especially in refractory tissues and large-animal models.

Current enrichment strategies, such as non-specific poly(A) capture, offer limited signal-to-noise improvement and fail to effectively isolate viral signals. Furthermore, because conventional workflows have restricted RNA input capacities, processing large-scale tissue samples necessitates splitting total RNA into multiple, labor-intensive reverse-transcription (RT) reactions, which increases technical variability and constrains throughput.

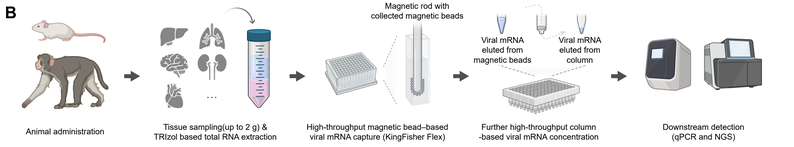
Here, we established CAPTURE, a sequence-specific mRNA enrichment platform that overcomes the "sparse signal" bottleneck in in vivo AAV screening. By consolidating gram-scale tissue inputs into a single-tube RT workflow, the platform significantly enhances detection sensitivity and data stability while maintaining exceptional biological fidelity (R > 0.95). CAPTURE performs robustly across diverse tissues and species, including NHPs, and supports the simultaneous analysis of multiplexed AAV libraries. This automated, high-throughput approach provides a powerful technical foundation for accelerating the directed evolution of next-generation gene therapy vectors.

METHODS

Capsid-Associated Probe-based Transcript Universal Recovery and Enrichment (CAPTURE)



Molecular process of gene-specific mRNA capture and concentration



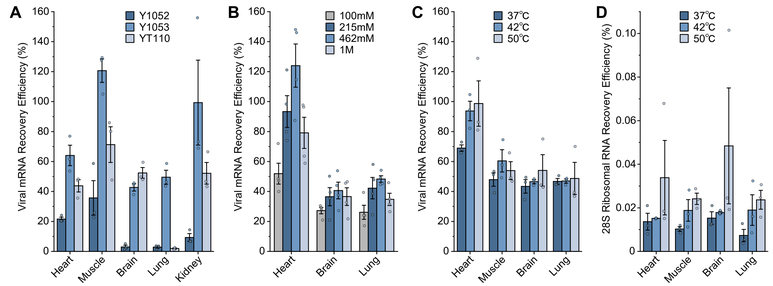
Experimental workflow for high-throughput sample processing

(A) The AAV capsid library is engineered with degenerate mutations flanking the peptide insertion site, creating unique flanking degenerate barcode sequences (fdBCs) that serve as molecular tags for variant identification and tracking. Following administration, tissue harvest, and total RNA extraction, viral mRNA is present at low abundance within a complex mixture of host RNA species (rRNA, tRNA, and mRNA). Biotinylated DNA probes are designed to hybridize specifically to these viral transcripts, which are subsequently captured using streptavidin-coated magnetic beads. After washing and elution, purified viral mRNA is recovered with substantially reduced background. These enriched transcripts are then concentrated and used as a high-density template for downstream reverse transcription and quantitative analysis. **(B)** AAV libraries are administered to mice or non-human primates, followed by tissue collection and total RNA extraction using TRIzol. The automated CAPTURE process is performed on a KingFisher Flex system, which manages the sequence-specific hybridization and magnetic bead handling. To maximize detection sensitivity, the eluted viral mRNA is further purified and concentrated into a minimal volume using column-based cleanup. This consolidated sample is then processed in a single-tube reverse transcription reaction, followed by quantification via qPCR or next-generation sequencing (NGS).

RESULTS

Hybridization Condition Optimization for Efficient and Specific Target Recovery

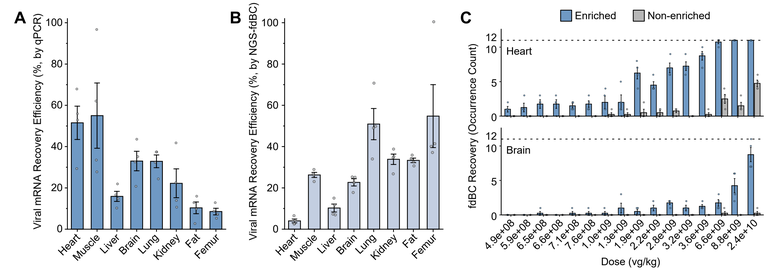
Probe selection and hybridization conditions were optimized to maximize viral mRNA recovery. Among tested probes, Y1053 consistently showed the highest recovery efficiency across tissues. Recovery efficiency was salt-dependent, reaching a maximum at 462 mM NaCl. Temperature optimization revealed that higher temperatures increased non-specific rRNA carryover, and 37°C was selected to balance recovery efficiency and specificity.



Systematic Parameter Optimization. **(A)** Comparative viral mRNA recovery efficiency across tissues using Y1052, Y1053, and YT110 probes (n=3). **(B)** Impact of NaCl concentration (100, 215, 462, 1000 mM) on capture efficiency (n=4). **(C, D)** Recovery profiling of target viral mRNA (C) and residual 28S rRNA (D) at 37°C, 42°C, and 50°C (n=3).

Enhanced Sensitivity for Sparse Viral Signals Across Murine Tissues

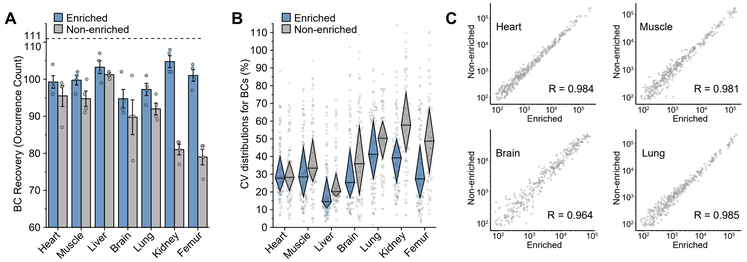
Following capsid library (R2) administration in mice, qPCR analysis revealed high-efficiency viral mRNA recovery, particularly in heart and muscle. NGS-based fdBC occurrence tracking demonstrated exceptional sensitivity in low-transduction tissues. However, in high-transduction organs, apparent recovery was constrained by fdBC saturation (max 12 per variant), causing a downward bias in signal quantification. AAV9 dose-gradient analysis confirmed that CAPTURE provides superior sensitivity and more reliable barcode recovery compared to non-enriched protocols.



Capsid Library (R2) Performance in Mice. **(A, B)** qPCR-based (A) and NGS-based (B) viral mRNA recovery across tissues (R2 library, n=4). **(C)** Dose-dependent fdBC recovery of AAV9 internal controls (max 12 fdBCs) in heart and brain (Enriched vs. Non-enriched).

Robust Variant Profiling with Broad Coverage, Stability, and High Fidelity

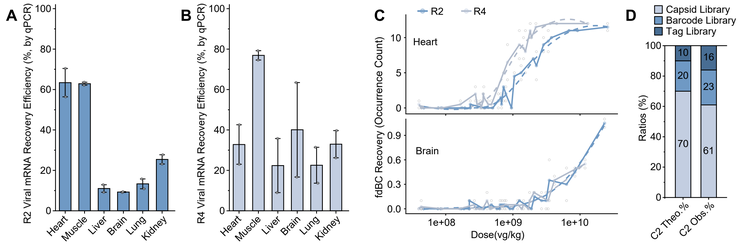
Using a 111-barcode library (LBC2), CAPTURE maximized detection coverage, with recovered BCs approaching the theoretical limit across all tissues. Enrichment significantly stabilized data, yielding lower CV distributions than non-enriched controls. High Pearson correlations (R > 0.95) confirmed that CAPTURE amplifies sparse signals while strictly preserving the original variant-abundance profile, ensuring high biological fidelity without introducing detection bias.



Detection Fidelity and Stability in Barcoded Pools (LBC2). **(A)** Unique barcode (BC) recovery counts (max 111) across murine tissues (LBC2 library, n=4). **(B)** CV distributions for BCs across biological replicates (n=4); diamonds indicate median and interquartile ranges (P25/P75). **(C)** Pearson correlation of BC read counts (Enriched vs. Non-enriched).

Gram-Scale Scalability and Multiplexed Library Enrichment in NHPs

The platform demonstrated exceptional scalability in NHPs, maintaining robust recovery across gram-scale tissue inputs (milligram-level total RNA inputs). High recovery was consistently observed across independent screening libraries (R2, R4). Analysis via fdBC frequency confirmed a linear dose-response, though measured recovery was constrained by fdBC saturation in high-transduction organs. Multiplexed enrichment showed NGS outputs closely mirroring predefined probe ratios, supporting parallel screening with minimal cross-interference.



Scalability and Multiplexed Enrichment in NHPs. **(A, B)** qPCR recovery efficiency of viral mRNA in NHP tissues (1–2 g input) for R2 (A) and R4 (B) capsid libraries. **(C)** Dose-dependent fdBC recovery (max 12) of AAV9 controls in NHP heart and brain. **(D)** Observed NGS output ratios vs. theoretical probe ratios for multiplexed library enrichment.

CONCLUSION

>> Scalable Gram-Scale Processing (Mice & NHPs)
>> High-Throughput Single-Tube Input Consolidation
>> Superior Sensitivity, Stability & Fidelity (R > 0.95)