Accurately Quantifying Transduction within Barcoded AAV Capsid Libraries via Tracking of Single-Molecule ID Tags

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Multiplexed barcoded capsid libraries have emerged as a powerful tool for high-throughput AAV engineering. Barcoded studies facilitate the accurate measurement of tropism across multiple capsids within a single animal, which is essential to understanding why certain capsids better transduce key delivery targets in vivo. However, many technical and biological artifacts complicate the reliable measurement of critical capsid properties, such as genome packaging and in vivo transduction efficiency. Notable artifacts include cross-packaging, template switching, and errors in DNA synthesis, which substantially reduce the quality of barcode-based library measurements, even when rare. To address these issues, we developed a paired library construction and data normalization strategy that flags potential "decoupling" between barcode identity and variant behavior. Incorporation of random DNA ID tags enables tracking of barcodes with synthesis errors, template switching, or cross-packaging followed by detection of outliers for each barcode-based measurement. Removal of barcode-ID pairs that significantly reduce the coefficient of variation in packaging-efficiency estimates then reliably prevents decoupled barcodes from contaminating subsequent analysis. Correcting barcode counts with this removal procedure, together with normalization according to internal positive and negative controls, improved the accuracy of packaging estimation on a reference set of wild-type and VP3-stop mutants. Furthermore, preventing decoupled barcode-ID pairs from propagating to downstream assays, such as in vitro and in vivo transduction, significantly sharpened these measurements. For example, in an in vitro transduction assay, filtering these decoupled barcodes reduced the false positive transduction rate among VP1 stops and narrowed the set of high-performing variants without impacting wild-type controls. This widely applicable approach significantly improves estimation of packaging and transduction in large, barcoded AAV libraries. Additionally, ID tracking enables estimation of new quantities of interest, such as the numbers of transfected and transduced cells per capsid variant. This improved estimation is highly relevant for the engineering of improved capsids, since reducing false positive rates substantially strengthens the power of models built with this data and increases the efficiency of later-stage capsid validation efforts.

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