Development of a molecular toolbox for the assessment of gene transcription during recombinant adeno-associated virus production

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Introduction

- Traditionally, production of recombinant AAV (rAAV) in HEK293 cells has relied on the transient transfection of three plasmids containing: **1)** AAV rep/cap, **2)** adenoviral helper genes *(*E2A, L4-22/33K, E4 and VA) and **3)** the gene of interest.
- RT-qPCR–based expression analysis offers a flexible and cost-effective alternative, particularly useful for monitoring inducible expression in producing cell lines and analyzing expression kinetics.
- To overcome the limitations of qPCR in accurately quantifying overlapping isoforms, we established a sophisticated dPCR approach that enables the individual detection of all *rep* isoforms.

Objective

To develop a molecular toolbox for the assessment of gene transcription during rAAV production in triple-transfection systems and packaging/producer cell lines.

Schematic representation rAAV production in a packaging cell line

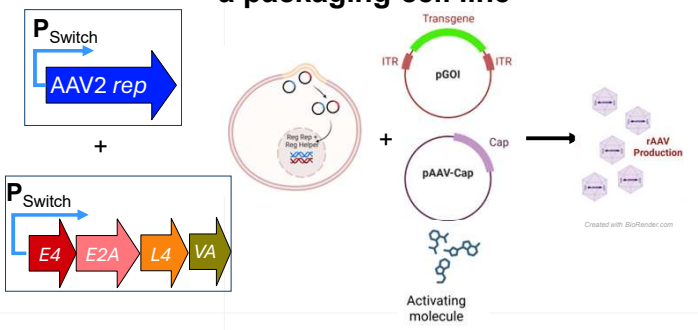


Figure 1. Plasmids carrying AAV2 *rep*, AdV5 E4, E2A and VA under the control of regulated promoters were stably inserted into NBX Xcell™ Eng-HEK293 cells. rAAV production is initiated after transfection of AAV *cap* and the GOI plus the addition of the activating molecule which initiates transcription of the regulated elements.

Primer design for rAAV production genes

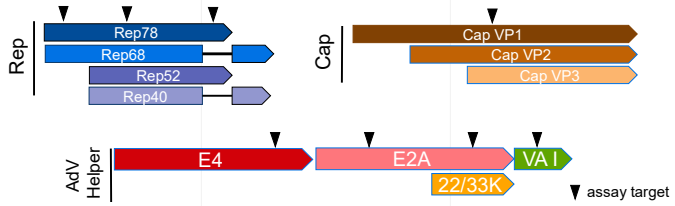


Figure 2. Target-specific primers were designed using transcript annotations and RNA-seq data.

q/dPCR assay workflow

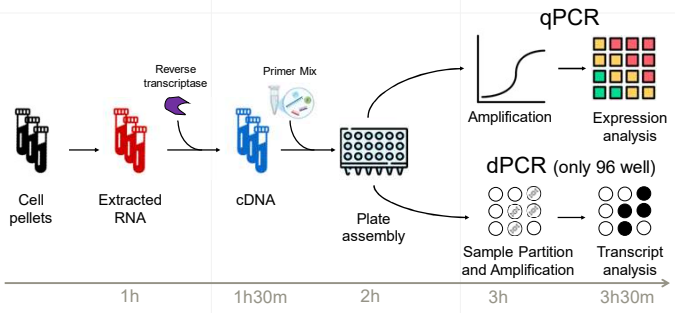


Figure 3. The workflow takes ~4 hours, is compatible with 96- or 384-well plates and can be automated from RNA extraction to data analysis.

References:

1. Vandesompele J, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3(7):RESEARCH0034. doi:10.1186/gb-2002-3-7-research0034

qPCR assay validation and control

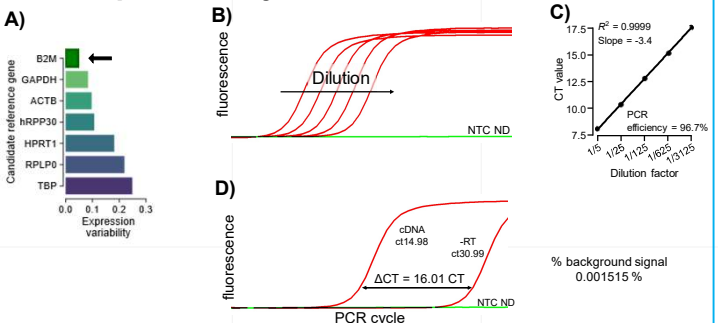


Figure 4. Plot showing the establishment of the assay and controls. **A)** Seven internal reference genes were screened to identify the optimal reference gene using the NormFinder algorithm [1]. **B)** Serial dilutions of primers were evaluated to determine PCR efficiency. **C)** Plot shoring the linearity of the serial dilutions. **D)** Primers were evaluated *in silico* against the NBX Xcell™ Eng-HEK293 genome and by qPCR with –RT and NTC controls to ensure specificity and exclude secondary structure artifacts.

Analysis of regulated components

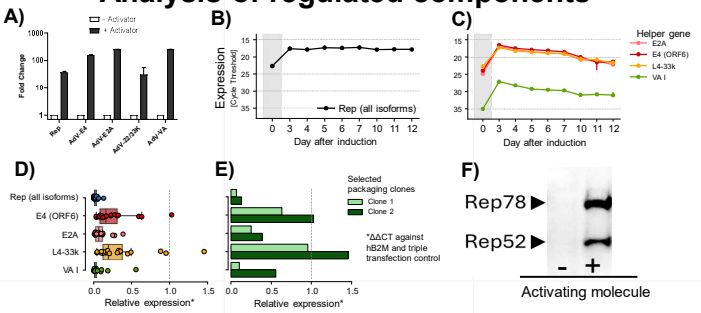
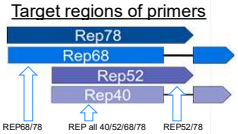


Figure 5. Analysis of the activation of regulated components in a regulated cell line. **A**) Transcription of regulated components in the presence or absence of the activating molecule shown as fold change over pre-activation. **B**) Transcription kinetics of *rep* after induction. **C**) Transcription kinetics of AdV helper genes after induction. **D)** Transcription of regulated components in 20 clones compared to triple-transfected cells. **E**) Transcription of regulated components in 2 rAAV packaging clones compared to triple-transfected cells. **F)** Western blot showing expression of rep protein species following activation with activating molecule.

Determination of *rep* isoforms

A) Target regions of primers



B) Model to calculate *rep* species transcripts

REP78 = % of total B (determined from colocalization analysis)
REP40 = B – C – A + REP78
REP52 = B – (A + REP40)
REP68 = B – REP78

C) Mixes of DNA fragments containing defined isoform ratios

ISOFORM	MIX1	MIX2	MIX3	MIX4
REP 40	10%	20%	30%	40%
REP 52	20%	30%	40%	10%
REP 68	30%	40%	10%	20%
REP 78	40%	10%	20%	30%

D) Calculated isoform ratios

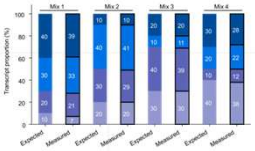


Figure 6. Relative transcription of rep isoforms determined using dPCR. **A)** Diagram showing the four rep species and relative location of the primers used. **B)** Model used to calculate rep species transcripts based on dPCR signals. **C)** Table showing mixes of pre-defined rep fragments to validate the model. **D)** Calculated rep isoform ratios determined from pre-defined rep species mixes.

Conclusions

- We developed a rapid and cost-effective qPCR-based approach to quantify gene transcription during rAAV production.
- Established targets include **rep, cap**, **AdV5 helper genes**, **E1A, E1B** and **internal reference genes.**
- An rAAV packaging cell line was shown to tightly regulate transcription of regulated components.
- Using fluorophore-labelled probes, dPCR enables precise quantification of overlapping rep isoforms within a sample and across samples.
- **This technology allows us to assess the dynamics of genes involved in rAAV production in NewBiologix's packaging and producer cell lines.**