

PCR Methods for the Characterization of AAV-based Drug Product from Titer to Potency

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Gene Therapy Analytical Development 2022

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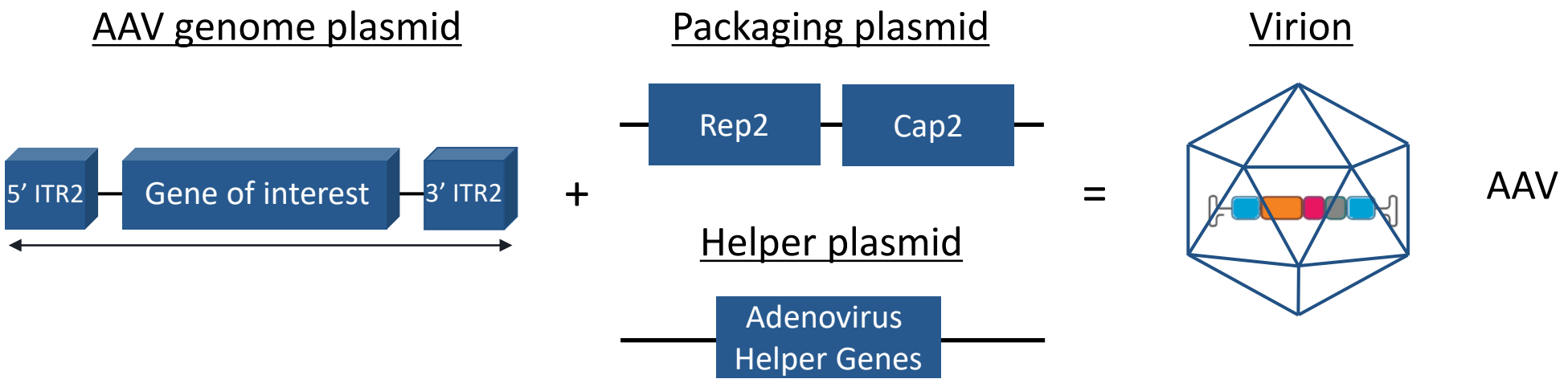
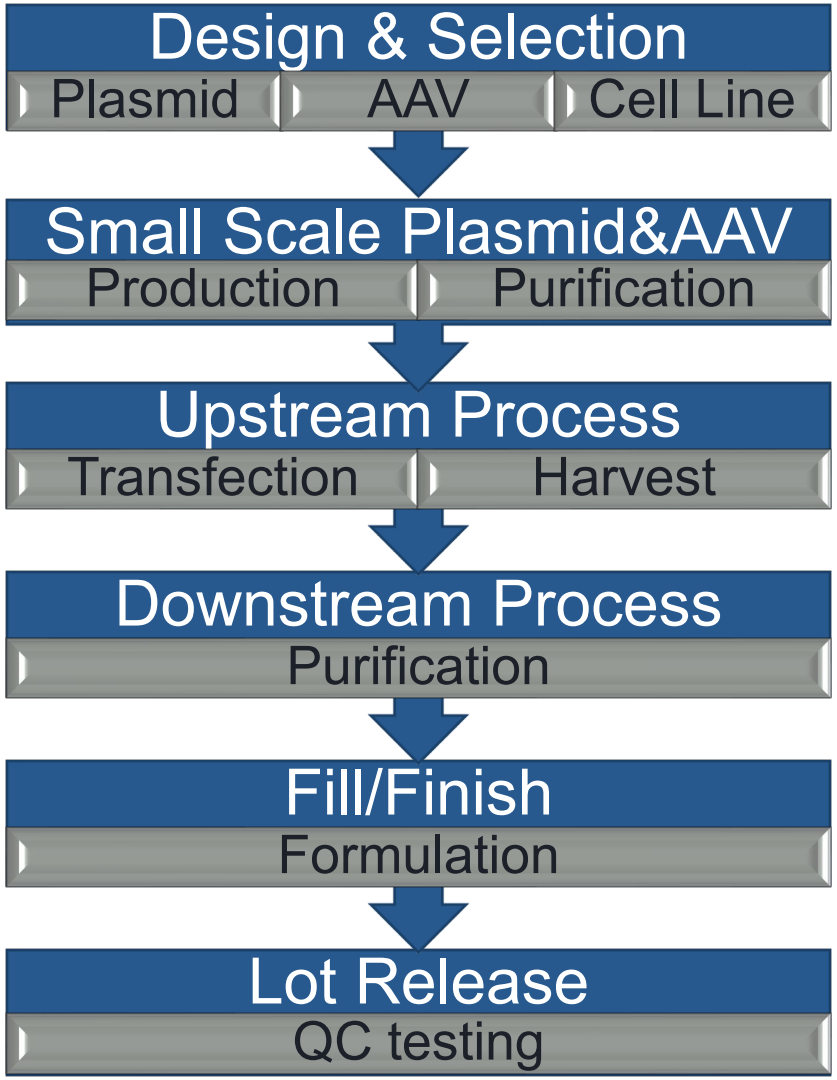
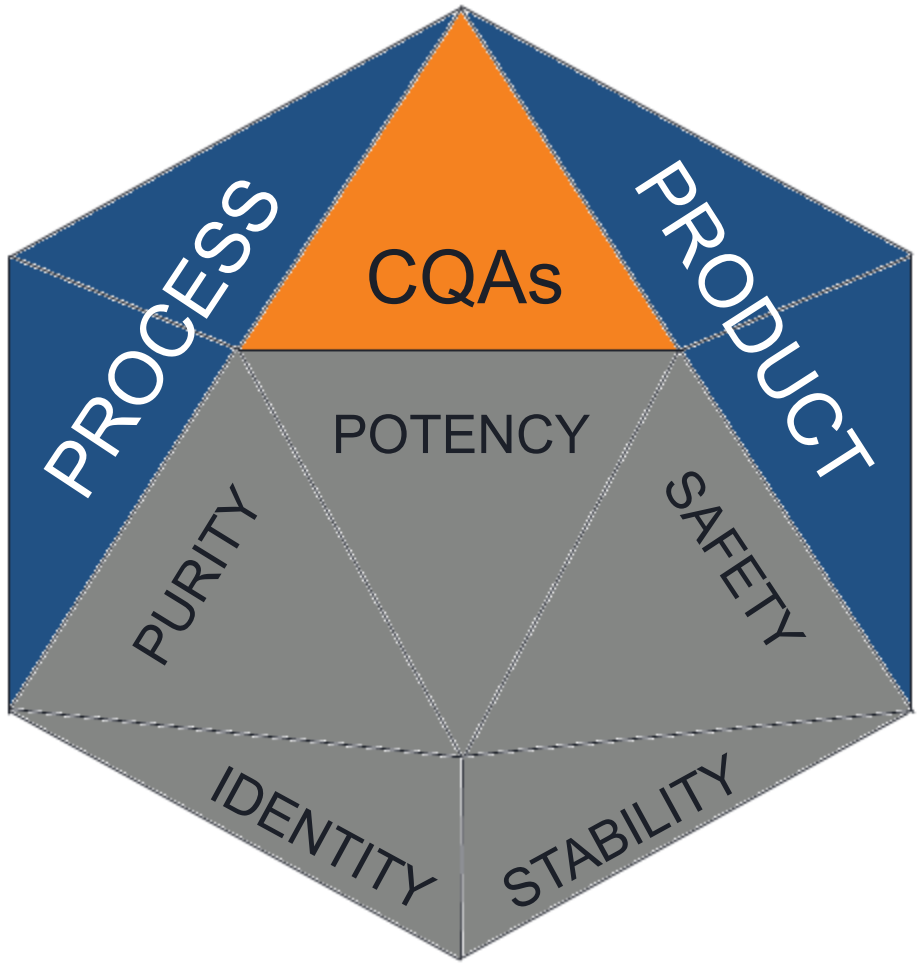
Adeno-Associated Virus (AAV) is an Ideal Vector for Gene Transfer

- AAV is a small (4.7 Kb), single stranded, non-pathogenic DNA virus
- Transduces both dividing and non-dividing cells
- Long-term transduction following a single administration
- Different AAVs exhibit distinct tropism for various tissues and organs in different species



rAAV CQAs Emerge as a Function of Vector and Process Design

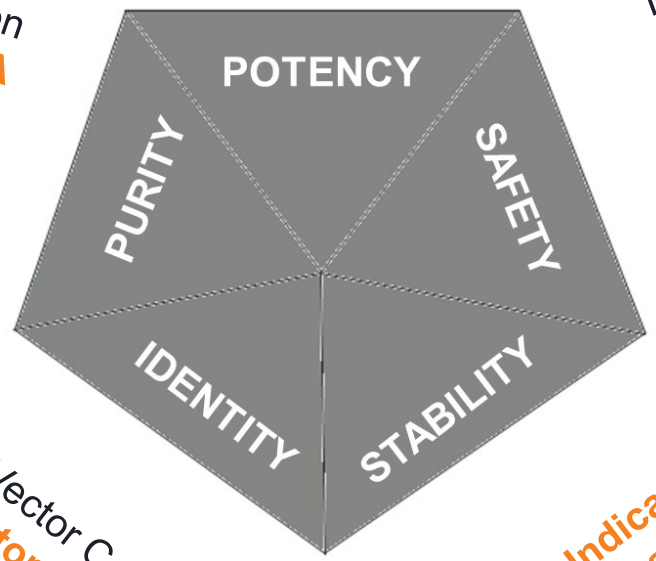




PCR Methods in a Typical QC Testing Panel for AAV Lot Release

VG Titer
Transduction Efficiency
Transgene Expression
Gene Editing
Trans-protein expression
Trans-protein function

OD260/OD280
Capsid Protein Concentration
Residual HCDNA
Residual helper DNA
Residual BSA
Residual HCP
Residual Benzonase
Vector Capsid
Aggregation



Viral Contaminants
Mycoplasma
Bioburden
rcAAV
Appearance
pH
Osmolality
Endotoxin
Subvisible Particles
Sterility

Vector Capsid
Vector Genome

Stability Indicating Assays

PCR Methods for Potency Evaluation

VG Titer
Transduction Efficiency
Transgene Expression
Gene Editing
Trans-protein expression
Trans-protein function

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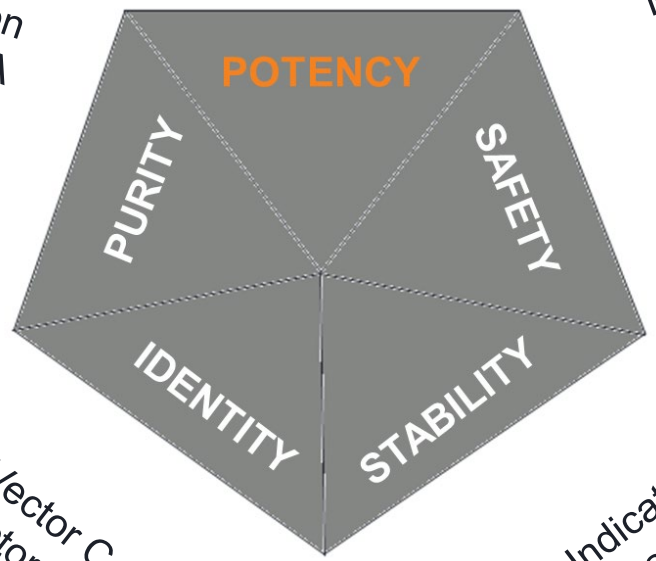

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Stability Indicating
Assays

Two Main Technologies for PCR Quantitation

SAMPLE

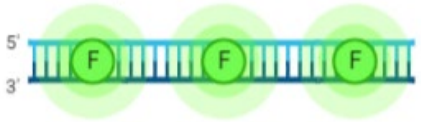
RNA

RT

DNA

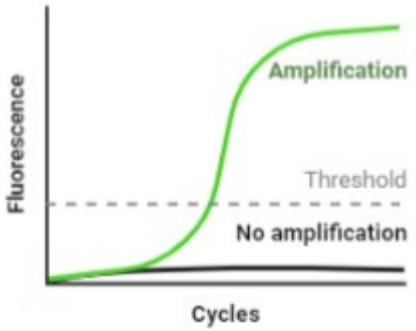
PCR

qPCR - fluorescence detection

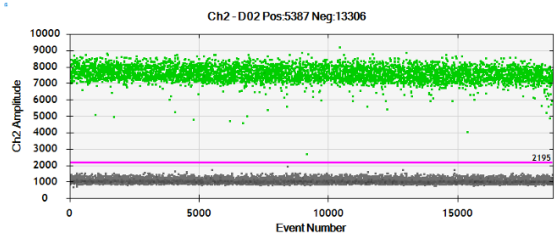


REPORTER DYE

REPORTER PROBE



ddPCR - direct counting of positive partitions adjusted for probability of co-occupancy with random distribution



$$c = \left(-\frac{\ln\left(\frac{N_{neg}}{N}\right)}{V_{droplet}} \right) V_{rxn}$$

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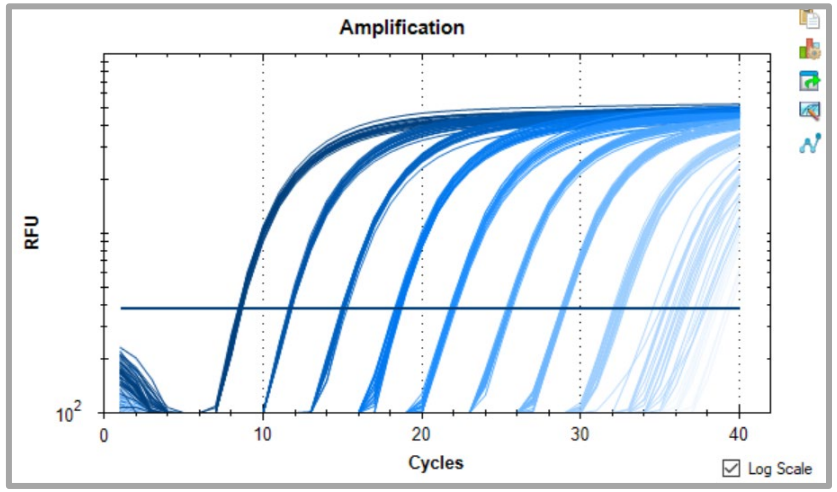
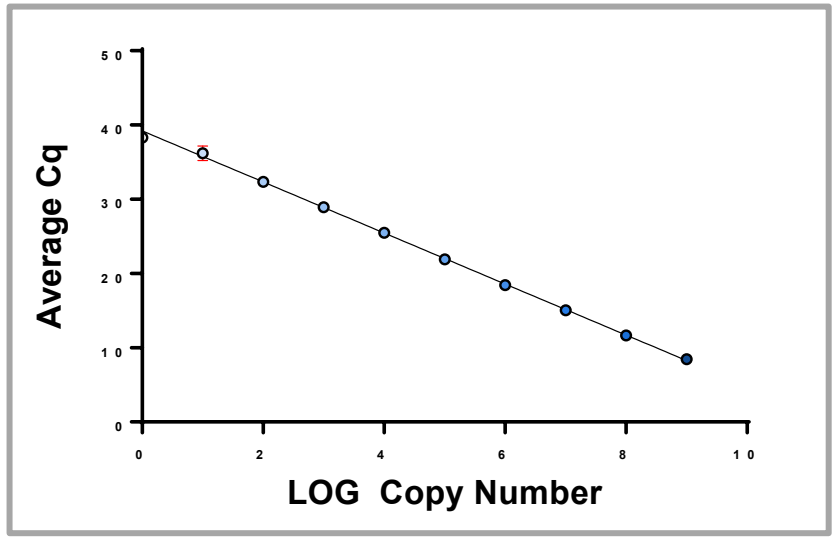
Real Time PCR Quantitation

qPCR[1]

1. Estimates target abundance against a calibration curve
   - Requires standards of known concentration
2. Precision depends on multiple variables from instrument to inhibitors
   - Measure 1.25-1.5-fold differences at best[2]
   - Assay precision deteriorates at low copy number (CN)
   - Sensitive to effect of inhibitors and differences in amplification efficiency
3. Dynamic range of ~9 logs
   - > ddPCR
4. Rare targets may not amplify
5. Multiplexing for ≥4 targets



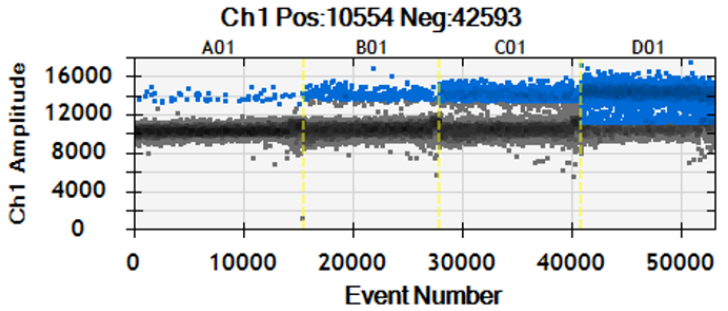


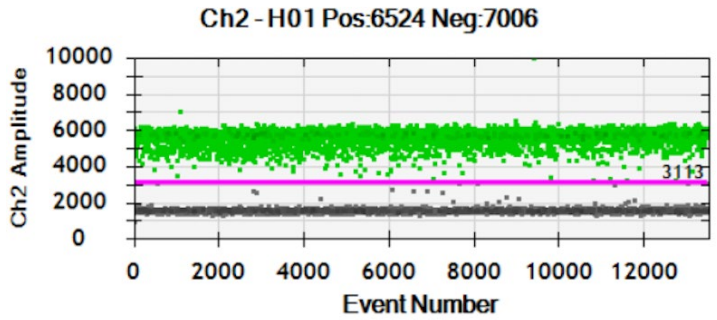
1 Bustin et al, Clin Chem 2009
2 Weaver et al., Methods 2010

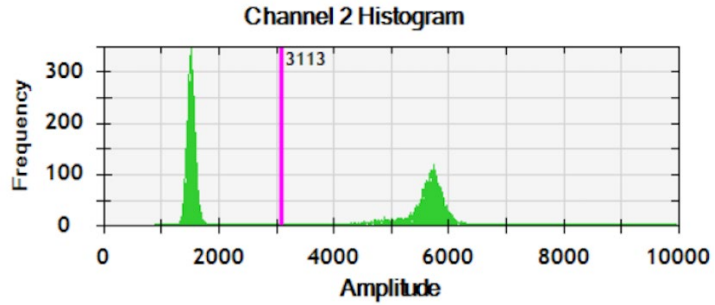
Droplet Digital PCR Quantitation

ddPCR[1]

1. Estimates target abundance from positive to total partition ratio[2]
   - Requires only known volume. Prior estimate of titer helpful but not essential
2. Precision ≈ # of partitions counts
   - Measure differences as low as <1.2-fold[3]
   - Assay precision drops at both ends
   - Tolerant of inhibitors
3. Dynamic range ≈ # of partitions
   - In practice < qPCR (~5 logs)
4. Sensitivity ≈ # of partitions
   - Frequency as low as 1/100K has been measured[4]
5. Only recently >2 channels for multiplexing







1 Huggett et al., Clin Chem 2013
2 Warren et al, Proc Natl Acad Sci USA, 2006
3 Whale et al, Nuc Acid Res 2012
4 Heyries et al Nat Meth 2011; Pekin et al, Lab chip 2011

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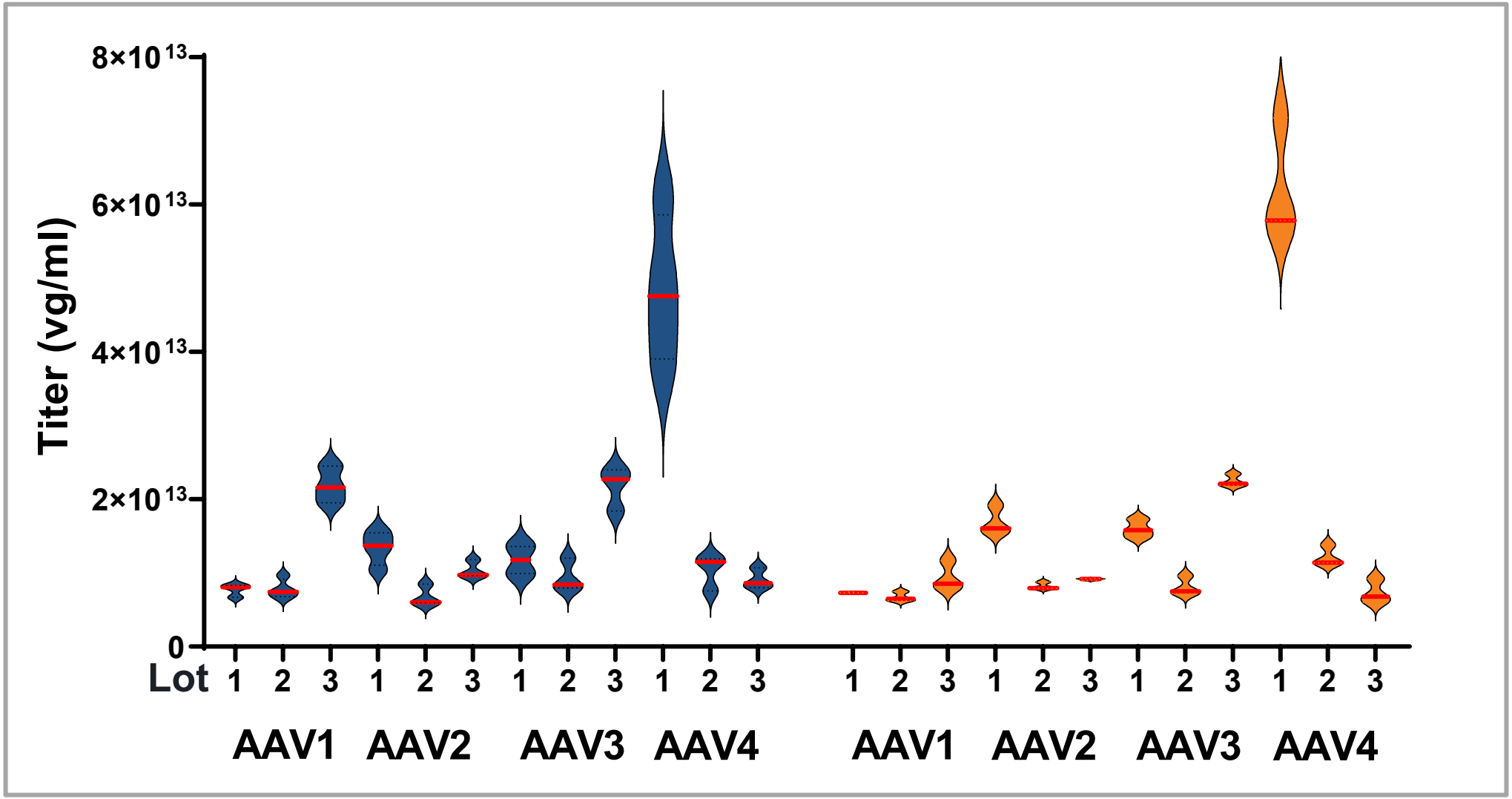
Case Study: Comparison of VG Titers by qPCR vs ddPCR



Average %CV by qPCR = 17% **Average %CV by ddPCR = 11%**

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Analytical Criteria for PCR based Method Development

SENSITIVITY

- # of copies accurately measured in the assay
- LOD ≤ LOQ
  - The concentration of template at 95%CI
  - ≥ 3 due to poisson distribution limits
  - Methods vary across industry
- LOQ
  - The lowest quantifiable concentration with acceptable precision and accuracy
  - Best determined empirically

SPECIFICITY

- Detects target sequence
- Does not detect any other sequences also present in the sample matrix

ACCURACY

- Differences between measured and known titers presented as fold change or copy # estimates

PRECISION

- Repeatability
  - Precision and robustness of the assay with the same samples analyzed repeatedly in the same assay
- Intermediate Precision
  - Variation in results between assay runs and/or laboratories
  - Test multiple reagent lots, instruments, operators

LINEARITY

- Dilutional
  - 3-5 non-zero points
- Cal Curve (qPCR)
  - ≥6 non-zero points
  - Run in duplicate
  - Identical matrix as samples
  - Linear regression on log transformed data

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Acceptance Criteria for PCR Based Titer

SENSITIVITY

- Acceptance criteria depend on assay
- For vg titer at LOQ %CV<25% and accuracy at 70-130%

SPECIFICITY

- NTC < LOD
- no interference in extracted gDNA
- Matrix effect evaluated on QC samples

ACCURACY

- QCs spiked in relevant matrix with established acceptance criteria
- Ideally QCs spanning the range of the assay

PRECISION

- CV ≤ 25% for intermediate precision
- Tighter for repeatability

LINEARITY

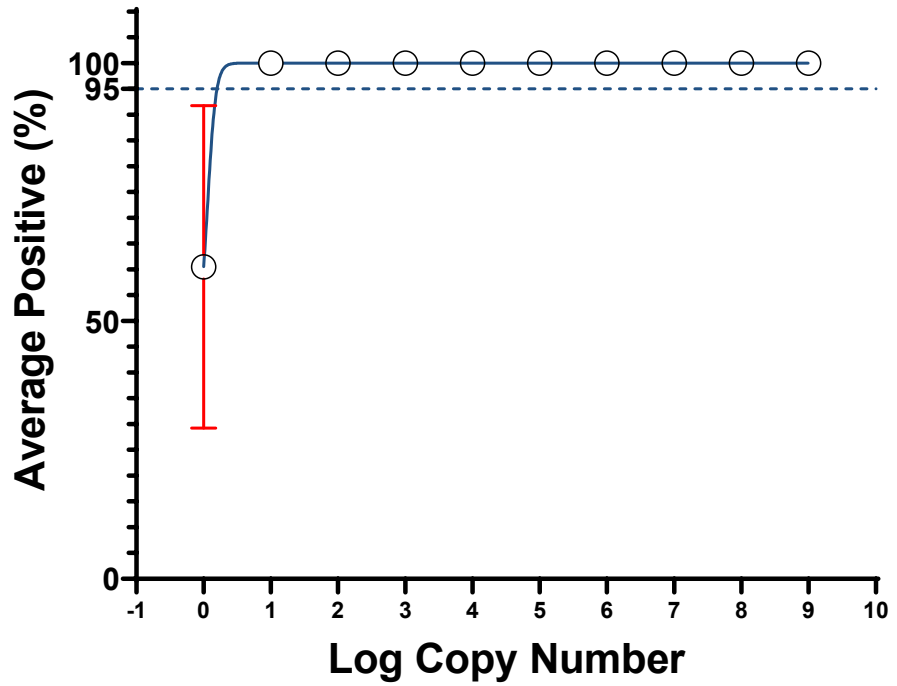
- qPCR amplification efficiency between 90-110%
- $R^2 \geq 0.980$

LoB/LoD and LOQ in qPCR

Methods to Establish LoD and LoQ

- LoB/LoD Calculation per CLSI guideline EP17
  - $LoB = mean\ blank + 1.645 \times \sigma_{blank}$
  - $LoD = LoB + 1.645 \times \sigma_{low\ concentration\ sample}$
- Problem: above equations apply to linear response
  - qPCR response is logarithmic and does not cross threshold for negative sample
- Alternative: establish LoD based on % positive response in replicate standard curves[1]
- For LOQ run the same 20 replicates and select the point with %CV<25% and accuracy 70-130%

LoD Based on 20 Replicate Std Curves



1 Forootan et al., Biomolecular Detection and Quantitation, 2017

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Other AAV VG Titer Considerations

qPCR vs ddPCR?

- Is ddPCR preferred over qPCR for regulatory filings?
  - No specific guidance but ddPCR has greater precision
  - Either assay should be acceptable if fit-for-purpose and properly validated
  - If feasible start with ddPCR in preclinical assay development to facilitate easy transfer to CMC

Titer Considerations

- Research material typically uses vendor titer with probe against ITR region
  - Overestimated concentration
- Confirm vendor titer with target gene specific assay
- Accurate titer assures equivalent dosing for reliable evaluation of AAV potency

Accurate vg Titer Critical for Accurate Potency Measurement

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Regulatory Guidance for Potency Testing of GT Products

What is Potency?

- Potency is defined as “the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result.”

  (21 CFR 600.3(s))

- Required for Lot Release, Stability, and Comparability

Guidance Documents

- FDA-2008-D-0520: Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products
- USP-NF<1032>: Design and Development of Biological Assays
- USP-NF<1034>: Analysis of Biological Assays
- ICH Q14: Analytical Procedure Development
- ICH Q2(R2): Validation of Analytical Procedure

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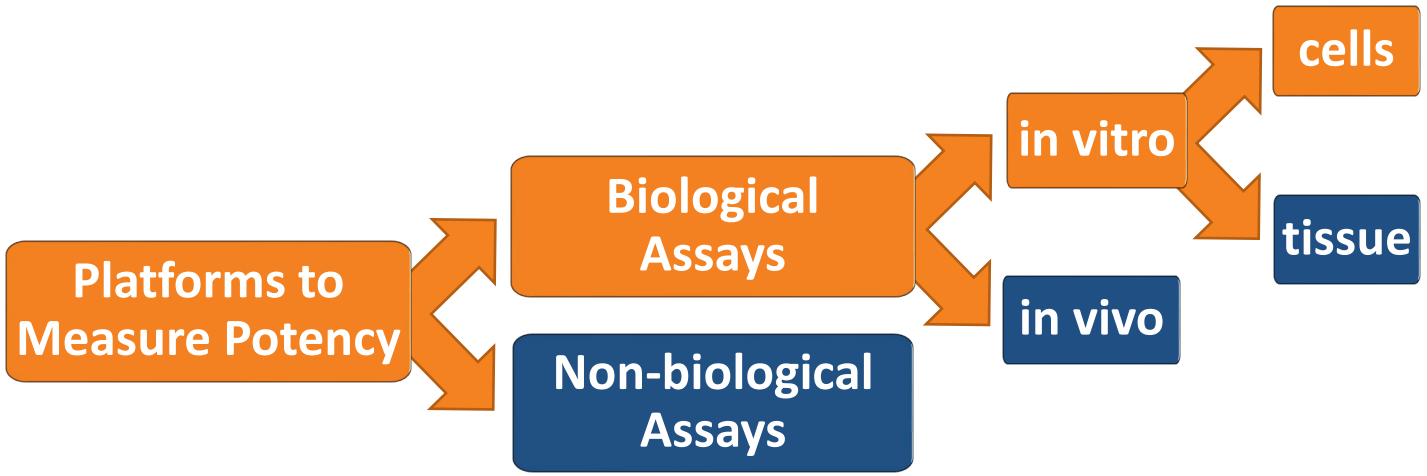
Potency of AAV Drug Product

- A gene therapy vector relies on 2 main biological activities for its potency
  1. the ability to transfer a genetic sequence to a cell
  2. the biological effect of the expressed genetic sequence



EDIT-103 for Autosomal Dominant Retinitis Pigmentosa (adRP)

- An inherited autosomal dominant retinal disease leading to blindness in later life
- Symptoms:
  - Decreased night vision (nyctalopia)
  - Loss of peripheral vision (tunnel vision), and eventually significant decline in central vision
- No approved treatments

Normal vision

Tunnel vision

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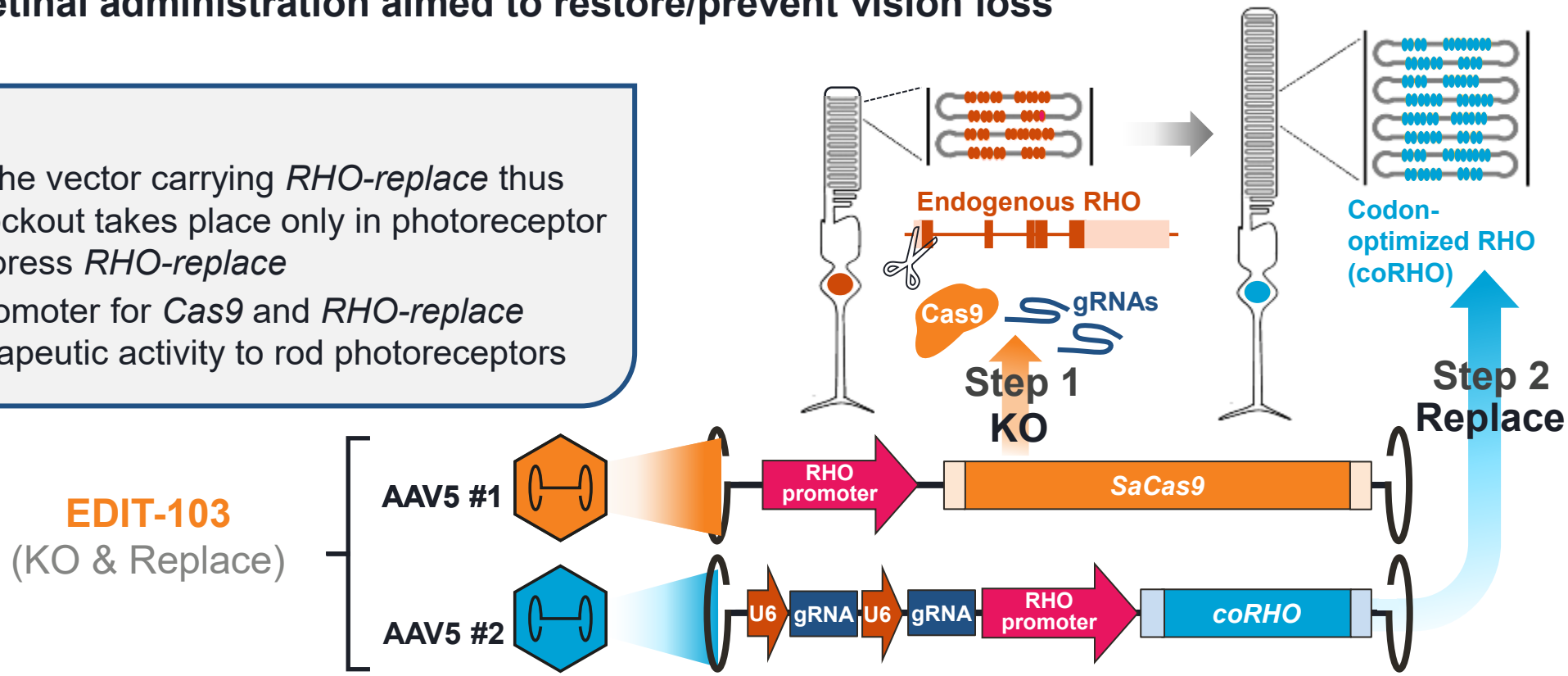
EDIT-103: Dual AAV-Based "Knockout and Replace" Therapeutic Strategy

- **Agnostic to any RHO mutation – thus will knockout any dominant gain-of-function rhodopsin mutant**
- **Step 1:** Both mutant and normal endogenous *RHO* will be knocked out in the treated area
- **Step 2:** Exogenous normal *RHO* (resistant to editing) will replace endogenous *RHO*
- **One-time subretinal administration aimed to restore/prevent vision loss**

Details:

- gRNA is on the vector carrying *RHO-replace* thus assuring knockout takes place only in photoreceptor cells that express *RHO-replace*
- The *RHO* promoter for *Cas9* and *RHO-replace* restricts therapeutic activity to rod photoreceptors



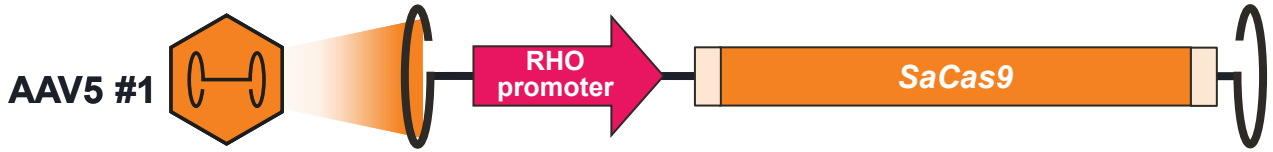
AAV, adeno-associated virus; Cas9, CRISPR-associated protein 9; coRHO, codon-optimized rhodopsin; gRNA, guide ribonucleic acid; KO, knockout; RHO, rhodopsin; SaCas9, *Staphylococcus aureus* Cas9.

Dual Vector Strategy Adds Complexity to Potency Assay Design

- EDIT-103 delivers two transgenes

Vector 1

- Expression of Cas9 endonuclease



Vector 2

- Expression of gRNA
- Expression of coRHO replacement at mRNA → **RT-ddPCR readout**
- coRHO expression-driven restoration to the structure/function of the photoreceptor

Vector 1 & 2

- Editing of both endogenous RHO alleles by the Cas9 endonuclease → **ddPCR readout**

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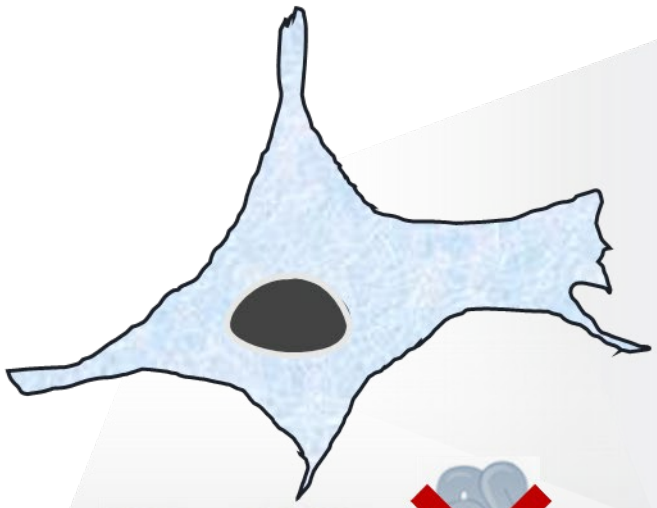
In-Vitro Potency Assay Platform Strategy

SUITABLE CELL LINE



Transducible by the chosen AAV serotype

Endogenous editing target

MEASURABLE FEATURES

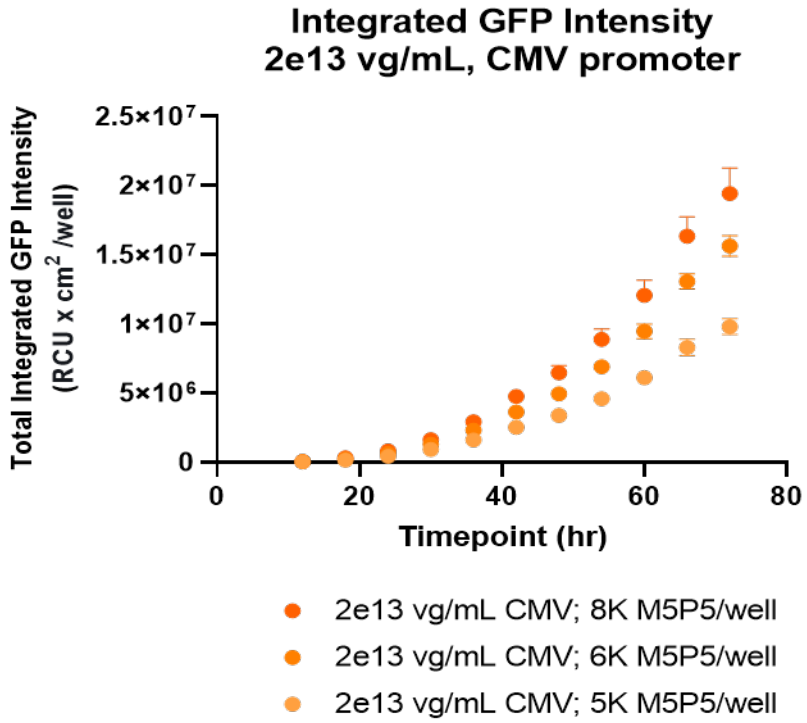
Transgene expression → Target editing

In-Vitro Potency Assay Platform Selection

In-Vitro Platform

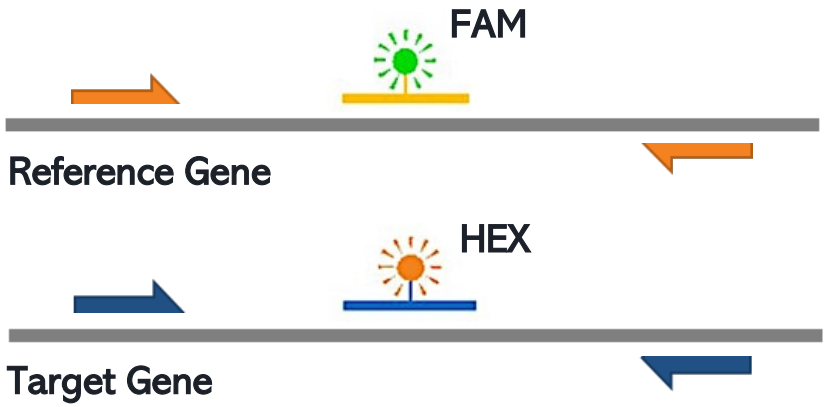
- HEK293T cells
  - contain but don't express endogenous *RHO* target
  - AAV5 transducible
- Additional cell line engineering required to enable the expression of transgenes under RHO promoter
- Cell culture conditions optimized for efficient transduction

AAV5 Transducibility

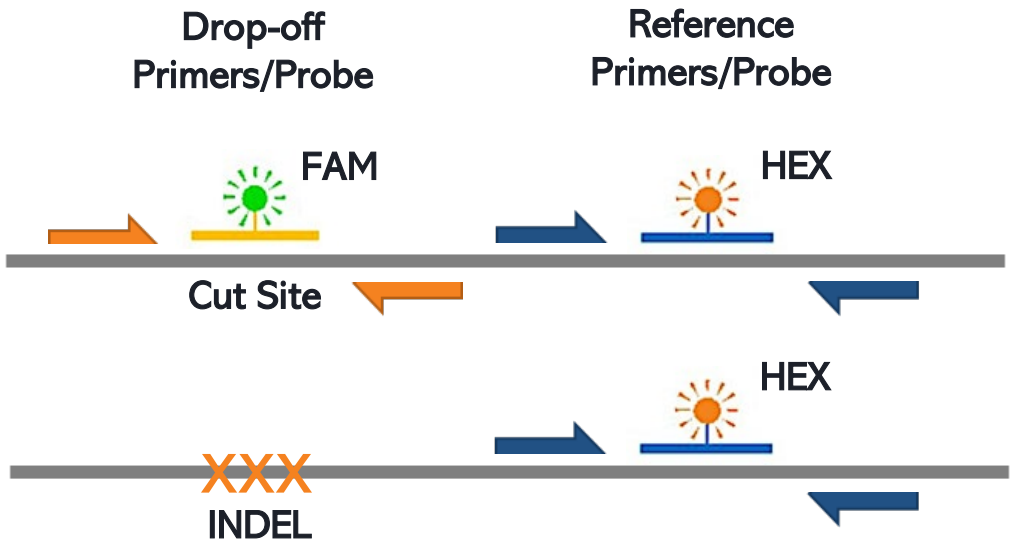


ddPCR Readouts for mRNA Expression and Target Editing

RT-ddPCR for coRHO mRNA expression

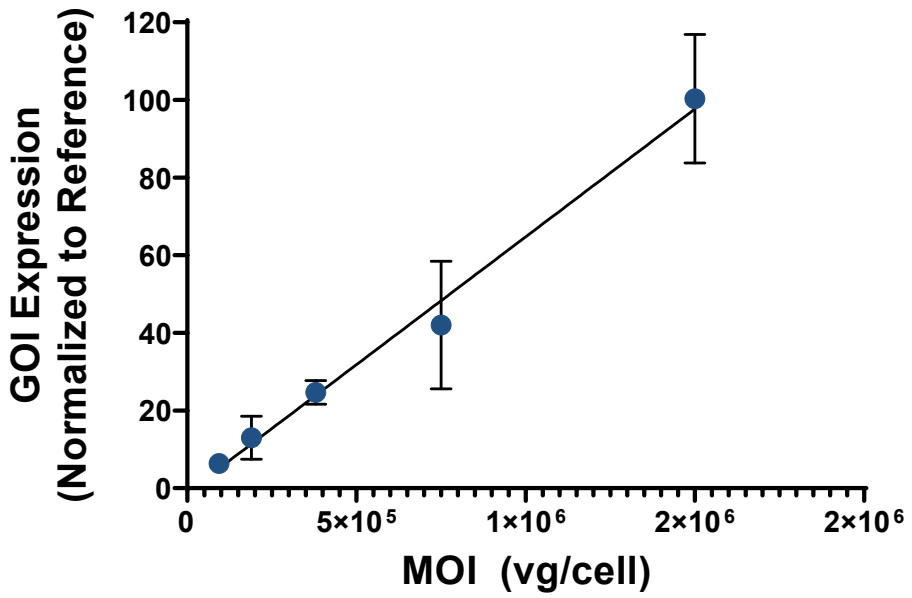


Drop-off ddPCR for RHO gene editing



Protein Replacement Potency Assay

Expression Response



R^2=0.9273 (n=3)

Linear Regression

Next Steps in Development

- Assay will be transferred to AD/QC for method optimization / validation
  - Establish Relative Potency
  - Optimize key assay steps
  - Establish system suitability criteria
    - Assay acceptance criteria
    - Sample acceptance criteria
  - Method qualification and transfer to QC for method validation

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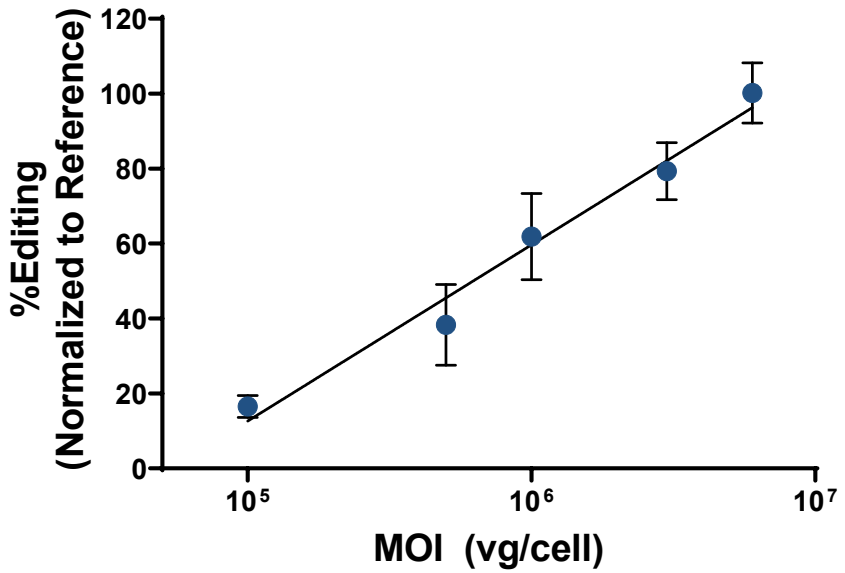
Editing Potency Assay

Editing Response



R^2=0.9117 (n=6)

Semilog non-linear fit (Log10 on x-axis)

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  - Establish Relative Potency
  - Optimize key assay steps
  - Establish system suitability criteria
    - Assay acceptance criteria
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  - Method qualification and transfer to QC for method validation

Summary

VG Titer Assay Development

- Don’t rely on ITR based vendor titers
- Develop target gene specific assays
- Test primers and probes *in silico*
- Design against cross-reactivity to matrix components and AAV cargo
- Select a platform that will easily transfer to regulated environment

Potency Assay Development

- Start potency assay development early!
  - Technically challenging
  - Slow TAT on assays
  - PCR based assays to prove editing and expression efficacy at IND
  - Functional potency for release of Phase 3 clinical material

Let the Full Lifecycle of the Program Guide Decisions in Assay Development

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Acknowledgements

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Jennifer Wendelken
Kate Zhang

* Alphabetical order according to last names

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