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
- Residual HCDNA
- Residual helper DNA
- Residual BSA
- Residual HCP
- Residual Benzonase
- Vector Capsid Aggregation

- Capsid Protein Concentration

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

- Vector Capsid
- Vector Genome

- Stability Indicating Assays

- Potency
- Purity
- Identity
- Stability

editas medicines
PCR Methods for Potency Evaluation

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

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

IDENTITY
- Vector Capsid
- Vector Genome

SAFETY
- Viral Contaminants
- Mycoplasma
- Bioburden
- rAAV
- Appearance
- pH
- Osmolality
- Endotoxin
- Subvisible Particles
- Sterility

STABILITY
- Stability Indicating Assays
Two Main Technologies for PCR Quantitation

- **qPCR - fluorescence detection**
  - Reporter dye
  - Reporter probe

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

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

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
   - 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

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 \(\approx \#\) of partitions counts
   - Measure differences as low as <1.2-fold\(^3\)
   - Assay precision drops at both ends
   - Tolerant of inhibitors

3. Dynamic range \(\approx \#\) of partitions
   - In practice < qPCR (~5 logs)

4. Sensitivity \(\approx \#\) 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
Case Study: Comparison of VG Titers by qPCR vs ddPCR

Average %CV by qPCR = 17%  Average %CV by ddPCR = 11%
# 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
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 ≥ 0.980
LoB/LoD and LOQ in qPCR

Methods to Establish LoD and LoQ

- LoB/LoD Calculation per CLSI guideline EP17
  - \( LoB = \text{mean blank} + 1.645 \times \sigma_{\text{blank}} \)
  - \( LoD = LoB + 1.645 \times \sigma_{\text{low concentration sample}} \)

- 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%

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\(^1\) Forootan et al., Biomolecular Detection and Quantitation, 2017
### 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**
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

• 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
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
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
Dual Vector Strategy Adds Complexity to Potency Assay Design

- EDIT-103 delivers two transgenes
  
  **Vector 1**
  - Expression of Cas9 endonuclease

  ![Diagram of Vector 1]

  **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

  ![Diagram of Vector 2]

- **Vector 1 & 2**
  - Editing of both endogenous RHO alleles by the Cas9 endonuclease → ddPCR readout
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**

![Graph showing integrated GFP intensity](image)

- Integrated GFP Intensity
- 2e13 vg/mL, CMV promoter
- Total Integrated GFP Intensity (RQ x cm²/well)
- Timepoint (hr)
  - 2e13 vg/mL CMV; 8K M5P5/well
  - 2e13 vg/mL CMV; 6K M5P5/well
  - 2e13 vg/mL CMV; 5K M5P5/well
ddPCR Readouts for mRNA Expression and Target Editing

**RT-ddPCR for coRHO mRNA expression**

- Reference Gene: FAM
- Target Gene: HEX

**Drop-off ddPCR for RHO gene editing**

- Drop-off Primers/Probe: FAM
- Reference Primers/Probe: HEX
- Cut Site: HEX
- INDEL: XXX

- Reference Gene: FAM
- Target Gene: HEX

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Protein Replacement Potency Assay

Expression Response

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
Editing Potency Assay

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

Next Steps in Development

Editing Response

- \( R^2 = 0.9117 \) (n=6)
- Semilog non-linear fit (Log10 on x-axis)
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
# Acknowledgements

<table>
<thead>
<tr>
<th>Mariacarmela Allocca</th>
<th>Lily Maxham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Racheal D’Souza</td>
<td>Mark S. Shearman</td>
</tr>
<tr>
<td>Pallavi Gambhire</td>
<td>Cristina Silvescu</td>
</tr>
<tr>
<td>Georgia Giannoukos</td>
<td>Adrian Timmers</td>
</tr>
<tr>
<td>Lily Li</td>
<td>Khushboo Undavia</td>
</tr>
<tr>
<td>Zoe Liu</td>
<td>Jennifer Wendelken</td>
</tr>
<tr>
<td>Eugenio Marco</td>
<td>Kate Zhang</td>
</tr>
</tbody>
</table>

* Alphabetical order according to last names*