A Platform Approach for Analytical Methods to Support Adeno-Associated Virus (AAV) Gene Therapy Products

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ABSTRACT

Recombinant adeno-associated viral (rAAV) vectors have been widely used for *in vivo* gene therapy with Luxturna™ and Zolgensma™ already approved by the FDA as commercial products. Even though a typical cGMP manufacturing of an rAAV gene therapy product consists of cell culture production (upstream), chromatography purification (downstream), and sterile filtration to fill / finish, unique manufacturing challenges for each unit operation warrant specific analytical methods to appropriately characterize and test these products.

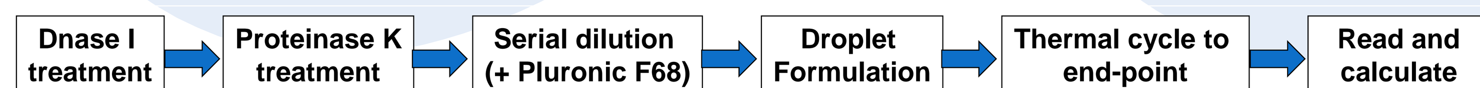
In order to quickly characterize and test rAAV gene therapy products, Charles River Laboratories (CRL) PA Biologics has established a platform approach for rAAV vector genome titer quantification, residual host cell DNA quantification and sizing evaluation, and replication competent AAV (rcAAV) detection, by leveraging real-time PCR (qPCR) and droplet digital PCR (ddPCR) technology platforms.

rAAV GENE THERAPY PRODUCTS GENOME TITER QUANTIFICATION BY ddPCR

As rAAV gene therapy products are designed to not replicate by themselves, genome copy number has been commonly used for clinical dosing. Historically, qPCR technology has been used to measure rAAV genome titer based a standard curve of a known quantity of plasmid DNA, which typically is created and quantified by individual laboratory without a reference standard. Approximately 15% - 20% variance in rAAV genome titer has been observed both between and/or within the same batch over time, when quantified by qPCR.

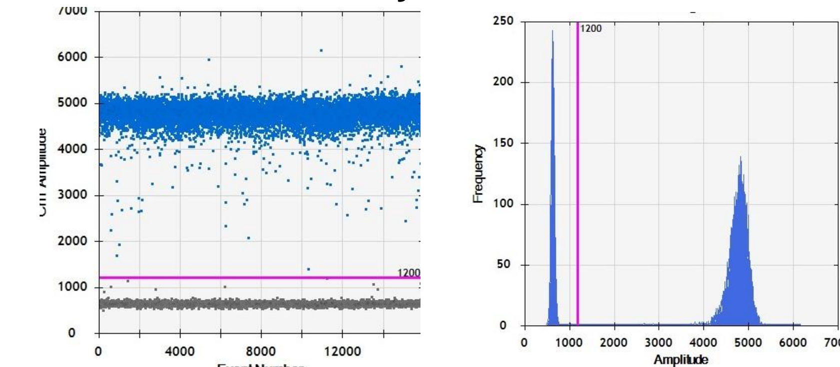
Recently, ddPCR technology has gained acceptance by gene therapy industries and regulatory agencies to measure rAAV genome titer. ddPCR utilizes water-oil emulsion droplet technology to fractionate a sample into ~20,000 droplets, with target-specific PCR amplification occurring in each individual droplet. Following PCR, each droplet is analyzed to determine the presence or absence of the target. The droplet data are then used to calculate the absolute target quantity by Poisson statistics. Absolute quantification by ddPCR eliminates the need for a standard curve, a major source of qPCR assay variability. In addition, ddPCR has a higher tolerance of matrix interference, as lower-efficiency PCR reactions still counted as positive droplets. Taken together, ddPCR technology is a highly suitable platform for rAAV genome titer quantification to support both in process and product release testing.

Schematic Sample Process for ddPCR-based rAAV Genome Titer Quantification



Evaluation of SV40 PolyA-specific Primer / Probe Set in ddPCR Assay

Observation:
- Good separation from positive vs negative wells with minimum "rain" observed
- Dilution yielding positive-to-negative droplet ratio closest to 1 is chosen for sample quantification



	Average genome titer (reported by ATCC using qPCR method)	Average genome titer obtained by CRL using ddPCR method	%CV of genome titer obtained by CRL using ddPCR method
AAV2 RSM (ATCC)	3.28×10^{10} vg/ml	3.94×10^{10} vg/ml (n=6)	5.1%
AAV8 RSM (ATCC)	5.75×10^{11} vg/ml	5.46×10^{11} vg/ml (n=6)	4.0%
AAV2 EGFP (CRL)	N/A	5.30×10^{9} vg/ml (n=5)	5.7%

Results:
- Purified and well characterized AAV2RSM and AAV8RSM obtained from ATCC resulted in the expected genome titer in ddPCR method, respectively
- Crude cell lysate containing AAV2 EGFP vector generated at CRL also resulted in a consistent genome titer in ddPCR method with less than 5.7% CV

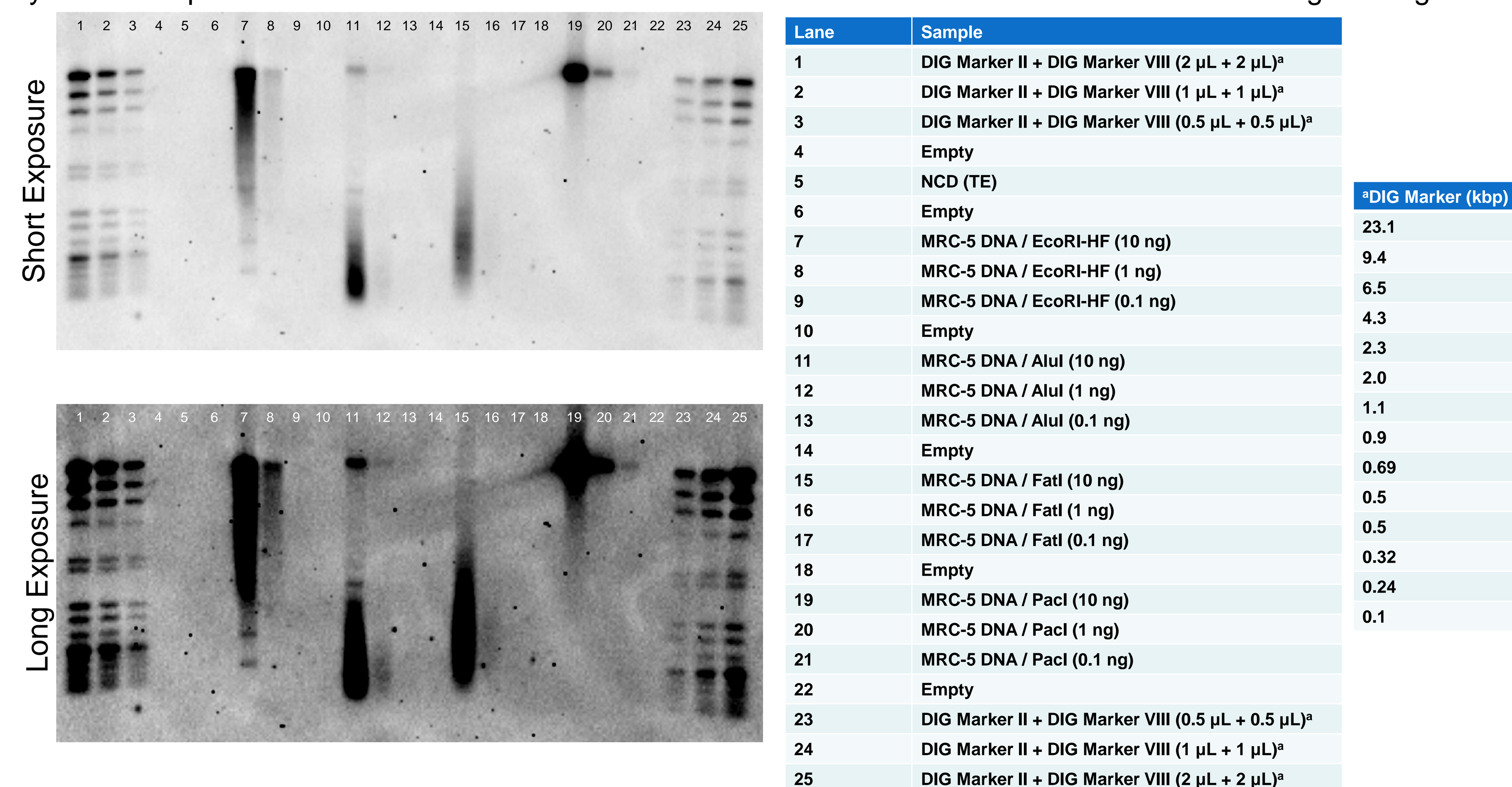
RESIDUAL HOST CELL DNA QUANTIFICATION AND SIZING EVALUATION BY qPCR AND SOUTHERN BLOT

Plasmid transfections into HEK293 cells is a widely used processes for cGMP manufacturing of rAAVs. rAAVs are more sensitive than many recombinant proteins, so purification options are often limited. This may result in a significant amount of residual host cell DNA in the final product, even after benzonase treatment followed by affinity and chromatograph purification steps. To ensure the biosafety of rAAVs, a target-specific qPCR assay is used to determine the total amount and the size distribution of residual host cell DNA in the final product.

Production cell line	Amplicon Size (bp)	Limit of Detection (LOD) (pg / 5 µl)	Limit of Quantification (LOQ) (pg / 5 µl)
HEK293	100	0.5	2.5
	250	0.5	2.5
	450	2.5	12.5

Assay Design and Generically Validated Results:
- Target a conserved region of 18s rRNA gene with 3 amplicon sizes by qPCR
- Monitor DNA extraction recovery and potential assay interference by qPCR signal of pre-spiked non-relevant plasmid control
- Generically validate LOD (indicated above per amplicon size) as greater than 95% of reactions shown as positive amplification in qPCR assay
- Generically validate LOQ (indicated above per amplicon size) by a combination of precision and accuracy for the dilution series of standard
- Determine the overall LOQ of qPCR assay by the initial sample volume subject to DNA extraction together with the final volume of purified DNA

While qPCR can quantitate selected sequence targets as representative of the entire genome, agarose gel electrophoresis can separate all host cell DNA present by size. The sensitivity of DNA staining in agarose gels alone does not allow for the detection of low levels (<10 ng) of residual host DNA. Southern blot analysis with hybridization probes and chemiluminescence detection allows for consistent detection levels of 1 ng – 10 ng.



Lane	Sample
1	DIG Marker II + DIG Marker VIII (2 µL + 2 µL)[a]
2	DIG Marker II + DIG Marker VIII (1 µL + 1 µL)[a]
3	DIG Marker II + DIG Marker VIII (0.5 µL + 0.5 µL)[a]
4	Empty
5	NCD (TE)
6	Empty
7	MRC-5 DNA / EcoRI-HF (10 ng)
8	MRC-5 DNA / EcoRI-HF (1 ng)
9	MRC-5 DNA / EcoRI-HF (0.1 ng)
10	Empty
11	MRC-5 DNA / AluI (10 ng)
12	MRC-5 DNA / AluI (1 ng)
13	MRC-5 DNA / AluI (0.1 ng)
14	Empty
15	MRC-5 DNA / FatI (10 ng)
16	MRC-5 DNA / FatI (1 ng)
17	MRC-5 DNA / FatI (0.1 ng)
18	Empty
19	MRC-5 DNA / PacI (10 ng)
20	MRC-5 DNA / PacI (1 ng)
21	MRC-5 DNA / PacI (0.1 ng)
22	Empty
23	DIG Marker II + DIG Marker VIII (0.5 µL + 0.5 µL)[a]
24	DIG Marker II + DIG Marker VIII (1 µL + 1 µL)[a]
25	DIG Marker II + DIG Marker VIII (2 µL + 2 µL)[a]

[a]DIG Marker (kbp)
23.1
9.4
6.5
4.3
2.3
2.0
1.1
0.9
0.69
0.5
0.5
0.32
0.24
0.1

REPLICATION COMPETENT AAV SEROTYPE 2 (rcAAV2) ASSAY

Although the rAAV cGMP manufacturing processes have been designed to reduce the risk of replication competent AAV (rcAAV) generation, there is still a possibility of rcAAV present in the final product, due to a recombination event. Therefore, rcAAV testing is required by guidance documents to ensure the biosafety of the final product. Cells permissive for the target AAV serotype are infected with the product being tested and either wild type AAV virus (if available), or a chimeric AAV with a matching serotype as a positive control, in the presence of human adenovirus 5 (Ad5) as helper virus to support rcAAV replication in cell culture.

Assay Design of Replication Competent AAV Serotype 2 (rcAAV2)
- Develop rcAAV2 assay using HEK293 cells with wild type AAV2 (ATCC) and human Ad5 (ATCC)
- Perform 3 rounds of amplification on HEK293 cells to amplify any potential rcAAV2, with samples taken at each passage (arrows)
- Detect any amplified rcAAV2 by Rep 2 – specific qPCR assay

Sample (HEK293 cells)	P1 Copy Number	P3 Copy Number
Uninfected	16 copies / µl	5 copies / µl
Ad5 Only	29 copies / µl	43 copies / µl
wtAAV2 at 1 TCID50 and Ad5	22 copies / µl	4.9×10^{6} copies / µl
wtAAV2 at 10 TCID50 and Ad5	8.3×10^{5} copies / µl	1.5×10^{6} copies / µl

Results
- After 3 rounds of amplification on HEK293 cells, as low as 1 TCID50 wild type AAV2 can be detected by Rep 2 – specific qPCR assay

Ongoing Experiments
- Evaluate a serial dilution of rAAV2 EGFP vector in rcAAV2 assay for rcAAV concentration
- Generically validate rcAA2 assay as a limits test
- Adapt this platform rcAAV2 assay to other AAV serotypes

SUMMARY

- CRL PA Biologics has established the platform approach for product-specific analytical methods to quickly characterize rAAV gene therapy products, by:
  - Targeting a well conserved region in human cells as well as common sequences in rAAVs for qPCR and ddPCR assays
  - Using virus stocks and reference materials from ATCC to validate assays
- CRL PA Biologics can further customize this platform approach for client-specific analytical methods, by:
  - Designing Gene-of-Interest (GOI) specific primer / probe set for qPCR and ddPCR assays
  - Creating chimeric AAV positive controls with the intended capsid and screening cell lines to support transduction of rAAV with these capsid for serotype-specific rcAAV assay.

For more information please contact: Charles River Biopharmaceutical Services: Yan.Zhi @ CRL.com; Michael.Hantman @ CRL.com
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