Development of a Sensitive NanoLuc® Based Bioluminescent Assay for Real Time Measurement of rAAV Transduction

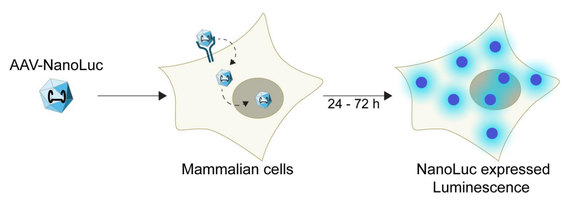
Trish Hoang, Rod Flemming, Vanessa Ott, Kara Machleidt, Jeff Nelson and Marjeta Urh

Promega Corporation, 2800 Woods Hollow Rd, Madison, WI, 53711

1. Introduction

Recombinant adeno-associated viruses (rAAV) are a widely used delivery vehicle for transgene expression in treatment of monogenic diseases. Key features that make rAAV a leading platform in gene therapy are tissue tropism, long-term transgene expression, and low immunogenicity. To further improve the target specificity, eliminate immunogenicity, and enhance efficacy, new engineered versions of rAAV are being designed along with optimization of transduction efficiencies in various cell types. During early development and optimization of novel rAAVs, surrogate protein expression (e.g., fluorescent proteins such as GFP) is used to track if the desired endpoint has been achieved. Despite being widely used, GFP expression is only measurable 48 hours post transduction due to its limited detection sensitivity. In this work, we describe a highly sensitive assay using NanoLuc® luciferase as a surrogate to monitor rAAV transduction in real time. NanoLuc luciferase is extremely bright, therefore allows for a very sensitive detection of gene expression. In addition, use of a cell permeable substrate enables live cell kinetic measurement of NanoLuc expression over extended periods of several hours or days. Moreover, assays can be performed on a standard luminometer. For the initial experiment, we generated AAV2 vector containing NanoLuc and transduced into a human cell line (20,000 cells per well) at different multiplicity of infection (MOI) in a 96-well plate. To monitor the expression of NanoLuc, luciferase substrate was added to the wells and luminescence signal was measured continuously for 60 hours. A time dependent increase in luminescence signal was observed 6 hours post transduction. Expression reached a plateau at 30 hours and the absolute luminescence signal was dependent on MOI. Once optimized, we demonstrate the utility of the assay for two applications: (a) rAAV transduction efficiency as a function of AAV serotype and cell type; (b) screening and detection of neutralization antibodies (nAb) against various rAAVs.

2. AAV-NanoLuc reporter: Concept, Format, and Workflow



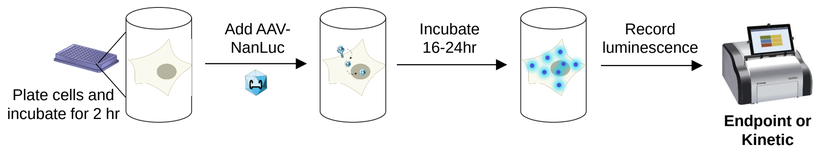
Concept of AAV-NanoLuc reporter to measure AAV transduction

NanoLuc is a small, bright, bioluminescence protein. NanoLuc reporter gene is encapsulated inside AAV virus. Different MOI of AAV are transduced into mammalian cells. Upon viral transduction, NanoLuc reporter gene is released and is translated to NanoLuc protein. Infected cells produce luminescent signal in the presence of substrate. The signal intensity is proportional to the transduction efficiency of AAV.

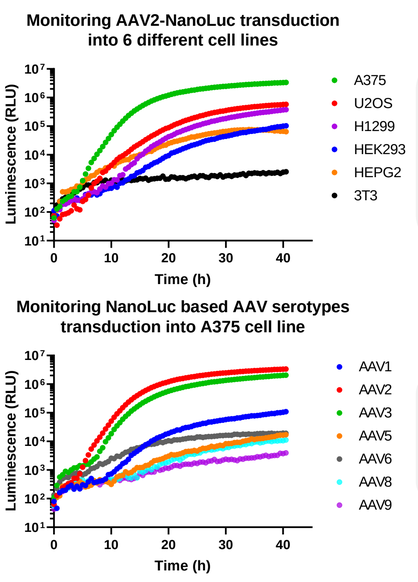
Components for AAV-NanoLuc transduction

- AAV-NanoLuc virus
- Mammalian cells
- Extracellular NanoLuc inhibitor
- NanoLuc substrate

Overnight protocol



3. Monitoring AAV transduction in real time



OBSERVATIONS

Among the tested cell lines, A375 is the best candidate for AAV2 based on the maximum signal, and the rate of luminescence signal increase over time.

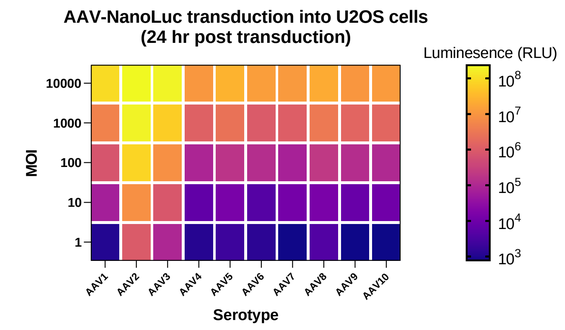
OBSERVATIONS

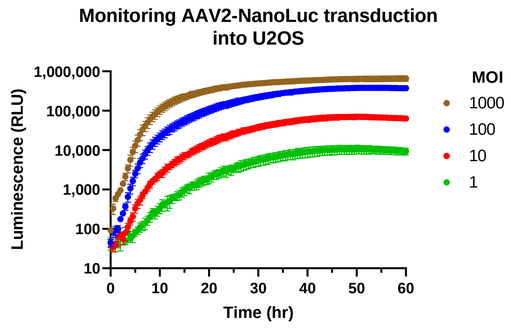
Kinetic measurement reveals that AAV2 and AAV3 are the best among AAV serotypes to infect A375 cells.

Key features of real time measurement of AAV transduction

- Kinetic measurement serves as a surrogate assay to monitor in real time transduction of AAV.
- Kinetic analysis would enable the discrimination of AAV transduction rate mediated by different engineered capsids variants.

4. Detection of AAV-NanoLuc at different MOI

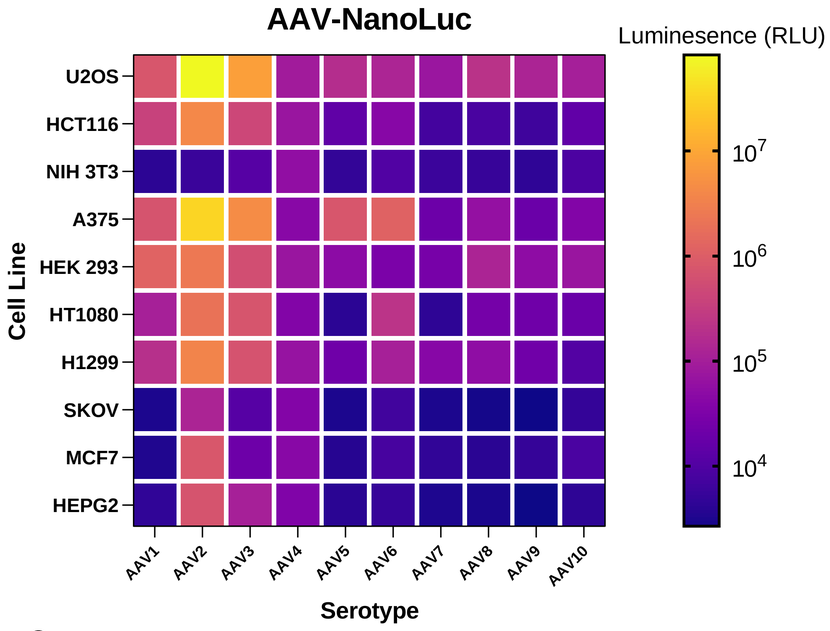




OBSERVATIONS

- Expression of NanoLuc is correlated with MOI.
- Significant expression of NanoLuc was detected at MOI as low as 10.
- Broad dynamic range of NanoLuc signal allows differentiation of transduction efficiency of AAV serotypes at MOI of 100.
- Kinetic traces show similar trends to end point detection.

5. Selectivity of AAV-NanoLuc toward different cell lines

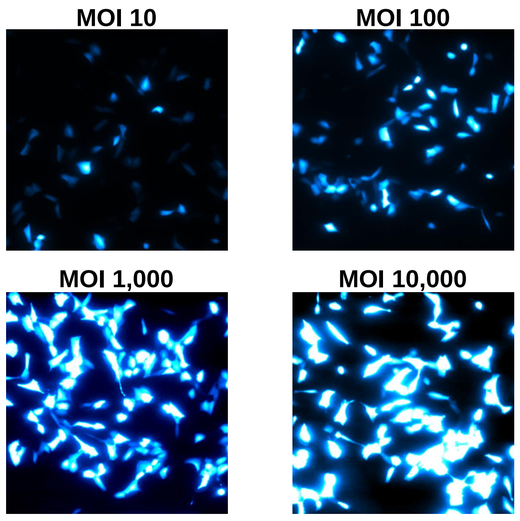


OBSERVATIONS

- A panel of AAV serotypes and a panel of cell lines that represents different tissue types were examined.
- Sensitivity of NanoLuc enables rapid testing of tissue tropism for each AAV serotype.
- AAV9, which is known to poorly transduce into cells, yields detectable signal even at 24 hours post transduction

5. Validation of NanoLuc expression by imaging

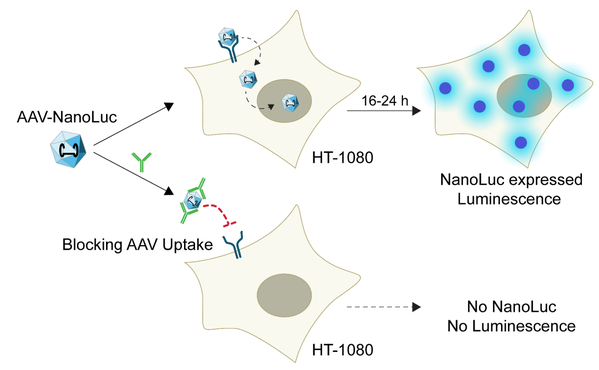
AAV2-NanoLuc transduction into U2OS at different MOI
Image taken at 24hr post transduction



OBSERVATIONS

- Luminescence images confirm expression of NanoLuc protein inside infected cells.
- Signal strength of NanoLuc in the plate-based assay is correlated to signal intensity observed by the luminescence images.

7. NAb assay: Concept, Format, and Workflow



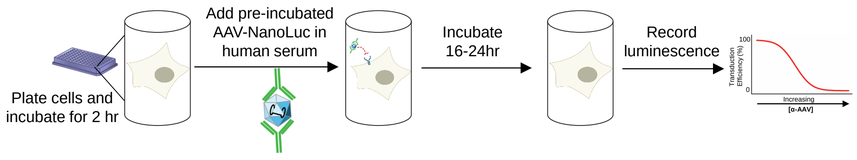
Concept of NanoLuc based Neutralizing Antibody (NAb) assay

NanoLuc based NAb assay measures transduction inhibition of AAV due to the presence anti-AAV antibodies in human serum. Antibodies block viral entry into infected cells and prevent NanoLuc expression. Reduction in luminescence would suggest the detection of anti-AAV antibodies in human serum.

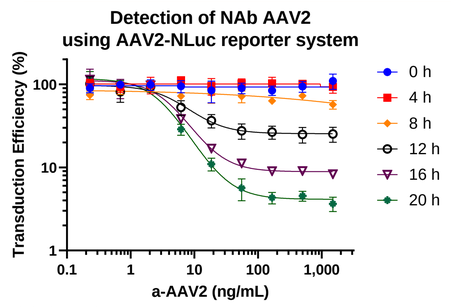
Components for NAb

- AAV-NanoLuc virus
- Human serum sample
- Extracellular NanoLuc inhibitor
- NanoLuc substrate
- Mammalian cells
- Negative serum control
- Spiked control α-AAV antibodies

Overnight protocol

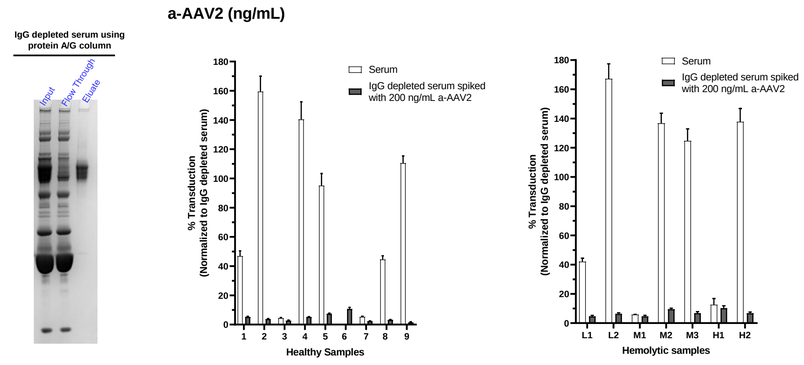


8. Detection of NAb in human serum samples



OBSERVATIONS

- α-AAV2 were diluted in PBS.
- Earliest point of detection is 16 hours post transduction.
- IC_{50} is 30 ng/mL.



OBSERVATIONS

- Protein gel shows IgG depletion in human serum.
- Depleted IgG serum serves as a negative control.
- Depleted IgG serum with a spike of α-AAV2 (200 ng/mL) serves as positive control.
- 9 healthy individual and 8 hemolytic individual were evaluated for NAb of AAV2.

9. Conclusions

We generated various AAV serotypes expressing NanoLuc luciferase and demonstrated capability for luminescent assay development for:

a) studying AAV tissue tropism

b) detecting anti-AAV neutralizing antibodies in human serum

Key features of AAV-NanoLuc reporter include:

- Simple and short workflow: NanoLuc expression can be detected 16-hours post transduction
- Endpoint and real-time kinetical measurement options
- Highly sensitive and tolerant to matrix interference when used for detecting NAbs
- High-throughput compatible
- Utilizes standard luminometer

Corresponding author: trish.hoang@promega.com

May 2022 | PS437

A fast and reproducible method to quantify full and empty adeno-associated virus

Trish Hoang, Phuoc An Vo, Kirsten Wingate, Vanessa Ott, Kara Machleidt, Jeff Nelson and Marjeta Urh

Promega Corporation, 2800 Woods Hollow Rd, Madison, WI, 53711

1. Introduction

Adeno-associated viruses (AAVs) have gained significant attention in the field of gene therapy due to their capacity for highly effective delivery of therapeutic genes into human cells. Recombinant AAVs are made in mammalian cell culture which produces three types of capsids: empty, partial, and full. Empty and partial capsids are considered impurities due to their lack of vector genome (vg) and associated therapeutic benefit. There are multiple technologies to assess AAV capsid status and impurities during AAV manufacturing, but most present challenges including low sensitivity, high cost, and laborious workflow. Therefore, we developed a faster and easier method by combining droplet digital PCR (ddPCR) and a nanoluciferase (NanoLuc)-based bioluminescent immunoassay technology (Lumit™) to quickly determine the percentage of full AAV. With ddPCR, we were able to accurately determine the copy numbers of vector genome in samples. We then applied Lumit™ Immunoassay technology and demonstrated that it can quantify intact viral particle (vp) AAVs with high sensitivity and broad dynamic range. The robustness and reliability of both assays (ddPCR and Lumit™) in combination enables the reproducible calculation of vg/vp ratio which equates to the percent of full AAV, particularly in crude samples. Our combined method serves as a valuable tool to provide measurement of identity and purity in AAV viral production.

Key features of ddPCR/Lumit™:

- Reliable quantification of AAV content ratio
- Single sample dilution used for ddPCR and Lumit™
- Easy incorporation of Lumit™ workflow into existing ddPCR workflow
- Quantifies percentage of full AAV from pure or crude samples
- Flexible format (96- or 384-well)
- Amenable to high throughput setting

2. Ratiometric method ddPCR/Lumit™ to determine percent full of AAV

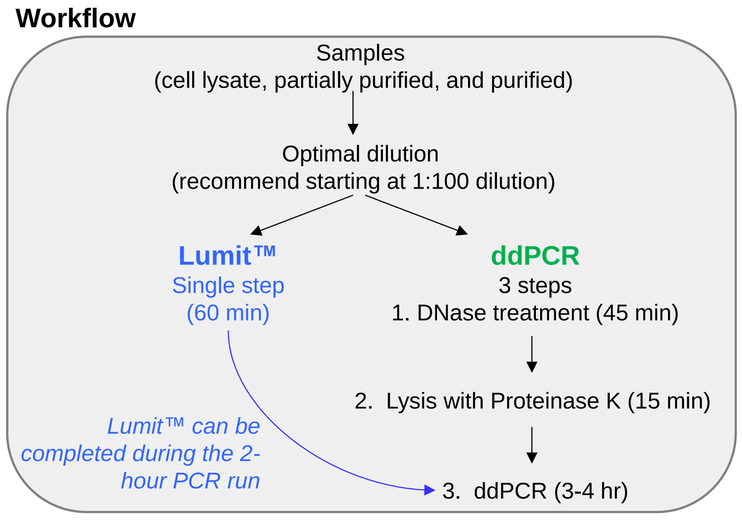
Rationale

ddPCR is widely used to quantify genomic titer which equates to the amount of full AAV. Lumit™ is used to determine viral titer which equates to the amount of total AAV virions. Lumit™, in conjunction with ddPCR, provides an estimation of percentage of full AAV in a given sample. Precision of both methods enables reliable estimation of AAV content ratio.

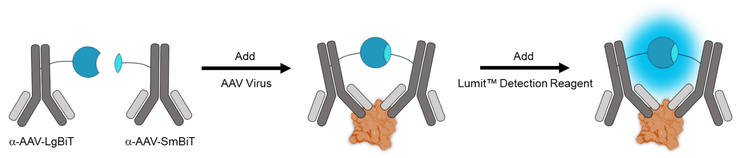
$$\frac{\text{ddPCR/qPCR/Dye}}{\text{ELISA/Lumit™}} = \frac{\text{gc/mL}}{\text{vp/mL}} = \frac{\text{Full AAV}}{\text{Total AAV}} = \%\ \text{Full AAV}$$

$$\frac{\text{ddPCR}}{\text{Lumit™}} = \frac{\text{Precise data}}{\text{Precise data}} = \text{Reliable Quantification}$$

Workflow



3. Lumit™ Immunoassay



Lumit™ Immunoassay technology can detect intact AAV capsids. In short, an antibody against a specific rAAV serotype is separately labeled with LgBiT and SmBiT. The presence of intact rAAV virion bridges the labeled BiT antibodies and facilitates complementation to reconstitute functional luciferase. Addition of substrate produces luminescent signal which is proportional to the amount of rAAV.

Assay components:

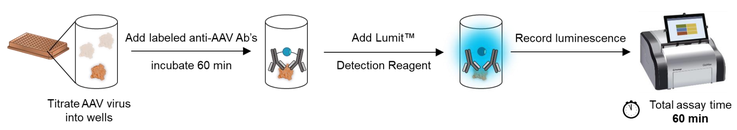
- AAV standard
- α-AAV-LgBiT
- α-AAV-SmBiT
- Lumit™ Assay Buffer
- Lumit™ Detection Substrate

Key features of Lumit™ Immunoassays:

- Easy and fast (single step protocol and 60 min assay)
- High sensitivity (detection of low viral titer)
- Broad dynamic range (3 – 4 logs)
- Flexible format (96- or 384-well)
- Homogenous and high throughput compatible

4. Comparison of Lumit™ and ELISA to quantitate intact AAV capsids

Lumit™ workflow



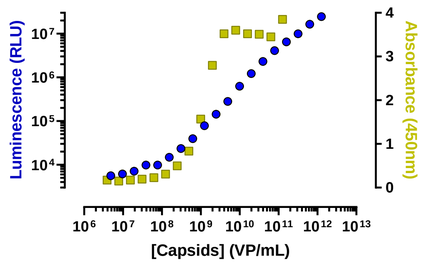
- Anti-AAV antibodies were purchased from Progen.
- AAV reference standards were purchased from Vigene Biosciences.
- ELISA kits were obtained from external sources.

Comparison of Lumit™ and ELISA workflow and assay requirements

	Lumit™	ELISA
Numbers of steps	1	5
Assay time	60 min	100 min
Assay temperature	Room temperature	37°C
Amount of virus	10 µL per well	100 µL per well
Signal stability after substrate addition	90 min	30 min

Comparison of Lumit™ and ELISA performance

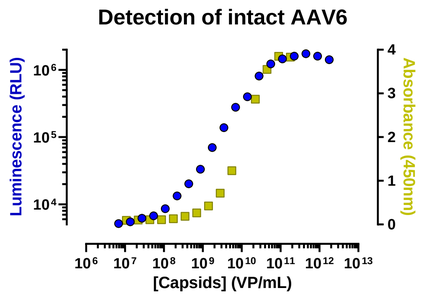
Detection of intact AAV2



	Lumit™	ELISA
LOD (VP/mL)	1.4x10^7	4.2x10^7
Dynamic Range (VP/mL)	1.9x10^7 to 6.4x10^{11}	6.2x10^7 to 2.0x10^9
MDD (VP/mL)	1.1x10^7	3.3 x 10^7

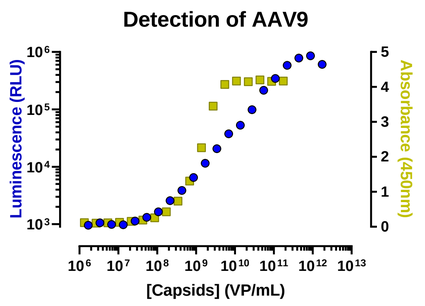
LOD = Limit of Detection
MDD = Minimal Detectable Dose

Detection of intact AAV6



	Lumit™	ELISA
LOD (VP/mL)	2.2x10^7	9.9x10^7
Dynamic Range (VP/mL)	5.4x10^7 to 5.6x10^{10}	1.8x10^8 to 2.2x10^{10}
MDD (VP/mL)	1.7x10^7	8.1x10^7

Detection of AAV9



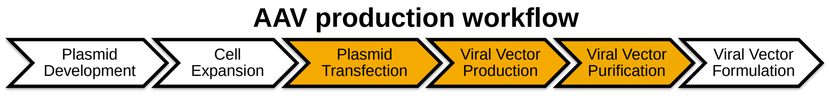
	Lumit™	ELISA
LOD (VP/mL)	3.3x10^7	1.8x10^7
Dynamic Range (VP/mL)	5.4x10^7 to 2.2x10^{11}	4.3x10^7 to 2.8x10^9
MDD (VP/mL)	2.8x10^7	1.5x10^7

OBSERVATIONS

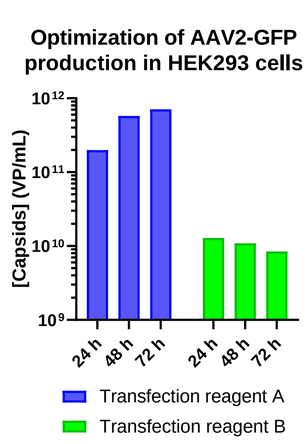
- Linear dynamic range of ELISA is limited.
- Lumit™ offers 3 – 4 orders of magnitude in dynamic range.

5. Applying Lumit™ to improve AAV production workflow

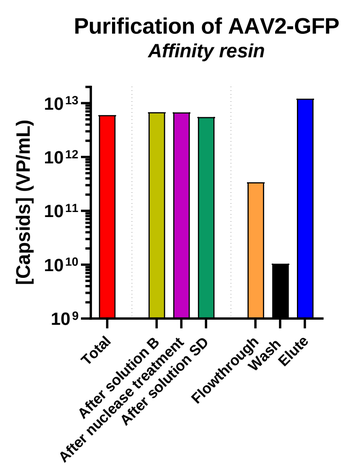
AAV production workflow



Optimization of AAV2-GFP production in HEK293 cells



Purification of AAV2-GFP
Affinity resin

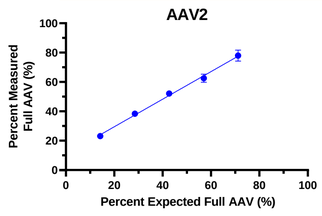


OBSERVATIONS

- Lumit™ can detect intact AAV in cell lysate and throughout purification process.
- Lumit™ can be used to screen transfection reagents and to optimize transfection conditions.
- Lumit™ can be used to optimize purification steps to improve yields.

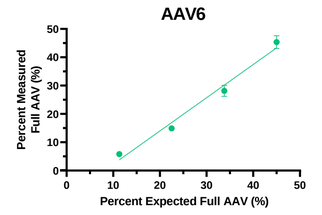
6. Determination of percent full AAV using ddPCR/Lumit™ ratio

AAV2



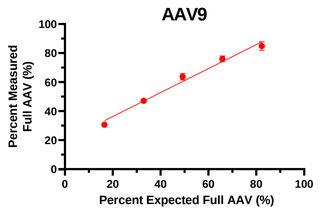
	Lumit™	ddPCR	ddPCR/ Lumit™
Intra-assay CV (%)	1.3	4.3	5.1
Inter-assay CV (%)	7.4	11.5	14.3

AAV6



	Lumit™	ddPCR	ddPCR/ Lumit™
Intra-assay CV (%)	4.0	5.2	6.8
Inter-assay CV (%)	7.7	15.2	14.6

AAV9



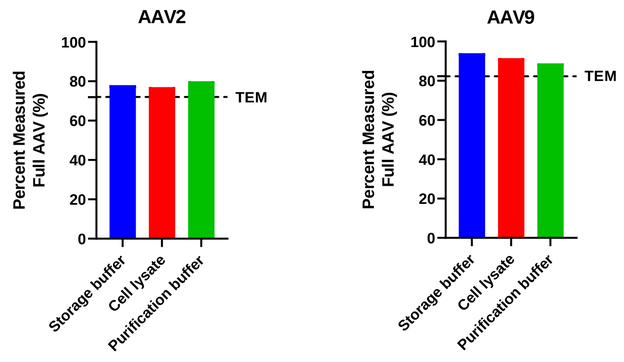
	Lumit™	ddPCR	ddPCR/ Lumit™
Intra-assay CV (%)	1.7	3.1	3.7
Inter-assay CV (%)	8.0	8.3	11.4

OBSERVATIONS

- Lumit™ demonstrates high precision with repeatability of 1-4% and reproducibility of ~8%.
- ddPCR/Lumit™ ratio shows improvement in both repeatability (3.7-8.1%) and reproducibility (11.4-14.6%) when compared to qPCR/ELISA ratio (4-24%, 11-36% respectively).

7. Determination of percent full AAV in cell lysates and purification buffers

- Full AAV reference standards were purchased from Vigene Biosciences.
- Full AAV samples were spiked into storage buffer, cell lysate or purification buffer.
- Percent measured full AAV were determined using ddPCR/Lumit™ ratio.
- A dash line is percent full AAV determined by TEM.



OBSERVATIONS

- Both ddPCR and Lumit™ methods can tolerate matrix effects, and are suitable for unpurified, in-process samples.
- ddPCR/Lumit™ method can be used in the enrichment process of full AAV virions during purification.
- ddPCR/Lumit™ method can be applied to optimize transfection conditions to increase full AAV populations.

8. Conclusions

We have developed a luminescent signal based homogeneous immunoassay platform (Lumit™) to accurately quantify intact AAV virions. The precision of Lumit™ enables the development of an improved ratio metric method (ddPCR/Lumit™) to calculate percent of full AAV in pure or crude samples.

- Lumit™ Immunoassays are rapid (60 min).
- A standard luminometer is needed for detection.
- ddPCR and Lumit™ combination is 4-5hr procedure.
- Both ddPCR and Lumit™ methods are high throughput compatible.
- ddPCR and Lumit™ are compatible with viral storage buffer, chemical lysis buffer, and purification buffers.
- ddPCR and Lumit™ can be used to improve full AAV yield throughout AAV production process.

If Interested:

1. Contact tailoredsolutions@promega.com for custom Lumit™ labeling and assay development services
2. Learn more about Promega's DIY Lumit™ Labeling Kit https://www.promega.com/products/immunoassay-elisa/lumit-immunoassays/lumit-immunoassay-labeling-kit-and-detection-reagents/?catNum=VB2500

May 2022 | PS436

Corresponding author: trish.hoang@promega.com