Development of a Potency Assay for an AAV-Encoded Transgene Regulated by Retina-Specific Promoters Using the *iLite*® Reporter-Gene Technology

Tovey M[2], Mayer U[1], Schwenkert M[1], Huang L[2], Lallemand C[2], Rodó J[1]

1. Svar Life Science AB, Malmö, Sweden
2. Svar Life Science AB, Paris, France

INTRODUCTION

Recent breakthrough innovations in gene therapy have brought us closer to treating many previously untreatable conditions. Gene therapy enables the replacement of defective genes with functional ones, aiming to restore normal function. While gene and cell therapies offer significant potential for addressing unmet medical needs, their development poses substantial challenges.

To help address this we have developed a reporter gene assay technology that allows for the quantification and assessment of therapeutic AAV vectors. This technology can be applied throughout drug development, including vector potency quantification and also the detection of neutralizing anti-drug antibodies (NAb).

Using our proprietary *iLite*® cGMP/GC reporter gene cell line, we have developed a custom cell line, subsequent assay-ready cells resulting in specific potency assays.

These assays have been tailored for AAV-encoded transgenes regulated by both photoreceptor-specific and retina-specific promoters, closely reflecting the mechanism of action of the transgene. One such assay, for the retina-specific protein, involves the stable co-transfection of ARPE-19-HPV-16 cells (derived from retinal pigmented epithelium) with a cGMP-responsive reporter-gene construct.

Additionally, the cells were stably transfected with Renilla luciferase (RL), allowing sequential quantification of FL and RL activity in the same well, thus normalizing FL activity relative to RL expression. This enhances assay reliability by making it independent of cell number and detecting non-specific effects.

The custom-made cell line described herein demonstrated a broad dynamic range in 4PL potency assays for AAV5-encoded transgenes without the need for live Adenovirus or external stimuli, offering a convenient, ready-to-use potency assay solution with low variability.

iLITE® cGMP/GC RESPONSIVE REPORTER CELL LINE

The *iLite*® cGMP/GC Responsive Reporter Cell line utilizes a host cell, stably transfected with an activation protein, where a secondary messenger molecule response functions as a measure of gene product activity.

The generation of the secondary messenger activates the formation of a transcription factor, binding to its designated promoter and inducing the expression of the firefly luciferase gene. The luciferase activity measured is directly proportional to the intracellular level of the secondary messenger which depends on the quantity of genetic cargo that has been delivered by the AAV transduction/infection (Figure 1).

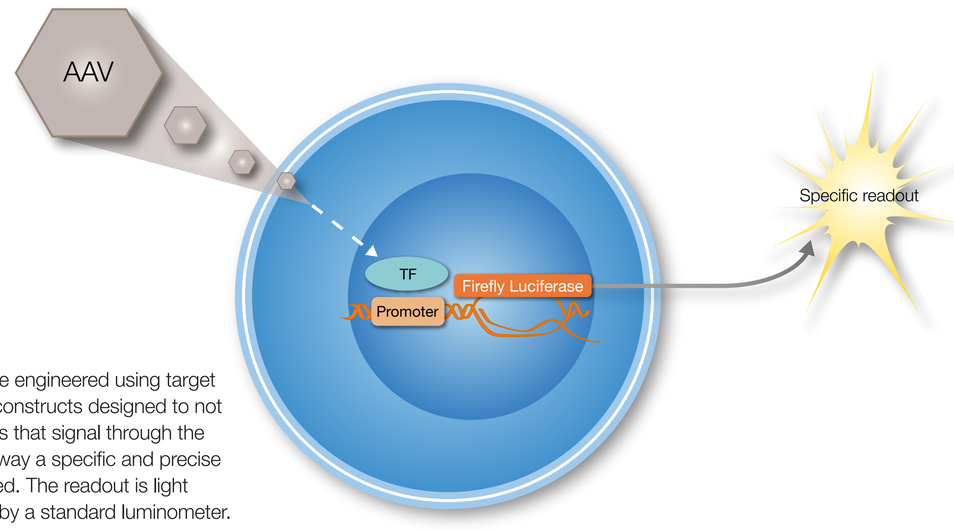


Figure 1.

The ***iLite*® cell lines** are engineered using target specific reporter gene constructs designed to not respond to other factors that signal through the specific pathway. This way a specific and precise assay can be engineered. The readout is light emission as measured by a standard luminometer.

SPECIFIC CELL LINE ACTIVATION

The *iLite*® cGMP/GC responsive assay-ready cells were developed for a rapid and sensitive assessment of guanylate cyclase activity. To establish specificity of the assay the cells were treated with either 8-Br-CGMP or 8-Br-cAMP.

As shown in the Figure the reporter-gene cells responded specifically to treatment with increasing concentrations 8-Br-CGMP as shown by an increased expression of the firefly luciferase (FL) reporter-gene expression. No increase in FL activity was observed when the cells were treated with increasing concentrations of 8-Br-cAMP. Moreover, the assay exhibited a dynamic range of 150-fold.

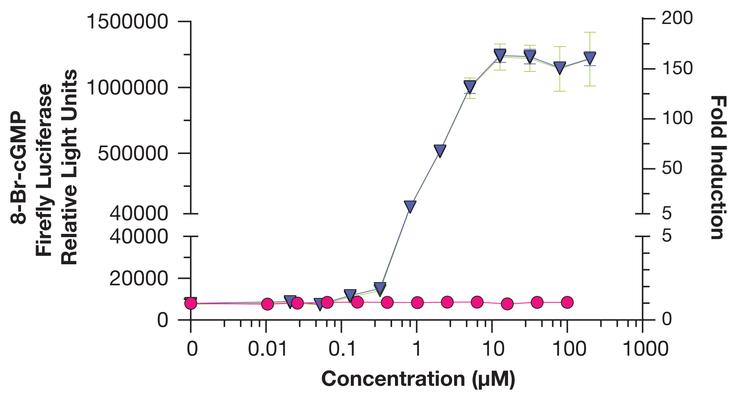


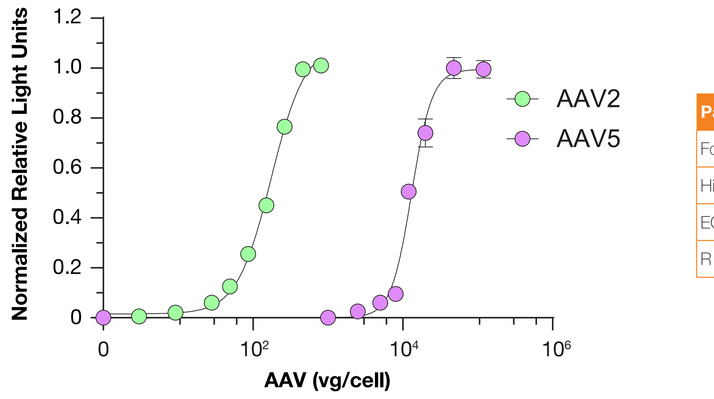
Figure 2.

Analysis of the AAV2 and AAV5 (0 – 1.1x10^7 vg/cell) serial dilution using *iLite*® cGMP/GC assay ready cells. The cells were stimulated with increasing concentrations of AAV2 or AAV5 vector over 72 hours in a humidity-controlled CO_2 incubator at 37°C. The data were presented as mean ± SD.

PARTIAL DOSE-RESPONSE CURVE

As the hook effect may impact the curve fitting an optimization of the dose-response curve has been done using a partial curve to correctly assess AAV titers. To this aim, a different starting dilution concentration was chosen for each vector. The range of the curve obtained from the stimulation with AAV2 or AAV5 vectors had a range of 0 – 809 and 0 – 1.2x10^5 vg/cell, respectively. These concentrations were prepared and incubated with *iLite*® cGMP/GC responsive assay ready cells.

The assay showed a high induction of the firefly reporter gene, allowing an accurate and specific serotype-independent determination of AAV titers.



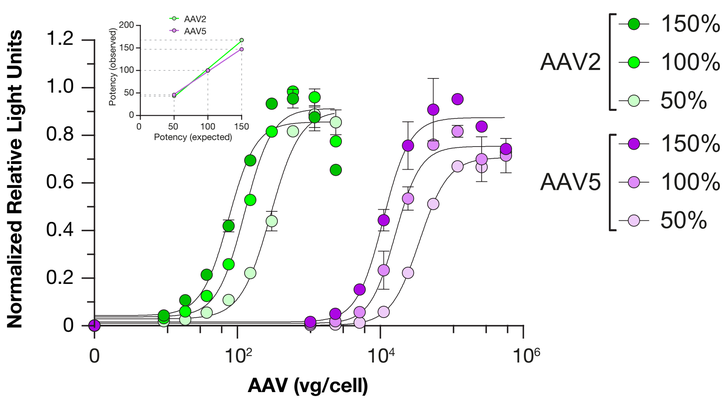
Parameter	AAV2	AAV5
Fold induction	>100	>100
Hill Slope	1.9	3.2
EC50	174	13140
R squared	0.99	0.99

Figure 3.

Analysis of the AAV2 (0 – 809 vg/cell) or AAV5 (0 – 1.2x10^5 vg/cell) serial dilution using *iLite*® cGMP/GC assay ready cells. The cells were stimulated with increasing concentrations of AAV5 vector over 72 hours in a humidity-controlled CO_2 incubator at 37°C. The data were analyzed using a 4-parameter logarithmic function, presented as mean ± SD.

POTENCY ASSAY

Potency assays can be developed and validated without difficulty and in a time-saving manner using the *iLite*® cGMP/GC assay ready cells. This technology supports the determination of AAV infectivity between different AAV lots as well as the comparison of various AAV manufacturing methods to transduce cells by detection of the different expression levels of the reporter gene.



	AAV2			AAV5		
Parameter	150%	100%	50%	150%	100%	50%
R squared	0.94	0.98	0.99	0.97	0.98	0.99
EC50 (measured)	76	127	292	11010	16190	35310
EC50 (expected)	85		253	10790		32380
Rel. Potency (measured)	168%		43%	147%		46%
Accuracy	89%		115%	102%		109%

Figure 4.

In this mock potency assay, cells were transduced using AAV2 or AAV5 with 10 equidistant MOI doses ranging for the AAV2 curve from 3.6x10^3 (150%), 2.4x10^3 (100%), and 1.2x10^3 (50%) vg/cell to 0 vg/cell or ranging for the AAV5 curve from 8.7x10^5 (150%), 5.8x10^5 (100%), and 2.9x10^5 (50%) vg/cell to 0 vg/cell. The assay was incubated for 72 hours in a humidity-controlled CO_2 incubator at 37°C and the luminescence was measured using Bright-Glo. The data were analyzed using a 4-parameter logarithmic function, presented as mean ± SD. The relative potency was calculated using the measured EC50.

NEUTRALIZING ANTIBODY DETECTION

The *iLite*® cGMP/GC responsive assay ready cells can be used in a functional assay to assess the potential inhibitor capacity of neutralizing anti-AAV2 and anti-AAV5 antibodies (NAb). The neutralizing effects of mixing human IgG with AAV2 or AAV5 vectors for 30 minutes could be shown in a dose-dependent inhibition of the expression of the reporter gene.

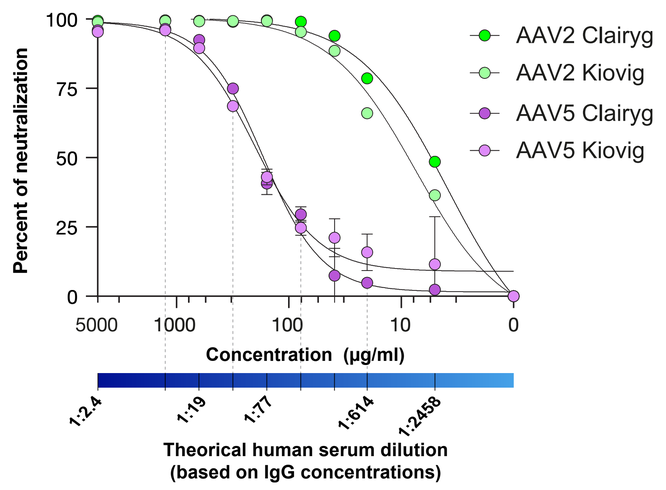


Figure 5.

Evaluation of functional anti-AAV antibodies. Two different pools of human immunoglobulins were serial diluted (IgG concentration shown in the blue bar) and incubated for 30 minutes before the addition to the *iLite*® cGMP/GC cells. Assuming an average value of 12 mg/ml IgG in human serum, the dilution grade is shown in the lower X-axis of the graph.

CONCLUSION

The face of the gene therapy field is becoming a reality to conquer so far untreatable conditions, but the AAV product development and approval processes still have multiple challenges that require substantial improvement. Here, we present a functional, robust and agile technology, *iLite*® cGMP/GC Assay Ready Cells (ARC) to facilitate the path towards the development of gene therapy products.

Svar's engineered *iLite*® cGMP/GC ARC overcome some of the hurdles associated with the development of AAV products during pre-clinical and clinical stages. This technology allows the evaluation of critical aspects such as AAV potency, AAV functionality or determination of pre-existing immunity, in an easy and time-saving manner. Moreover, the system can be adjusted depending on the potential application.

The data presented here indicate the following:

- **The *iLite*® cGMP/GC ARC assay tolerates a broad MOI range, establishing a sigmoidal 4PL curve fit with a large linear dose-response range.**
- **Due to its high sensitivity, our technology can be used in a responsive and reliable potency assay application. Differences in the AAV effectiveness between batches to transduce cells can be clearly determined and assessed through the different expression levels of the reporter gene.**
- **The *iLite*® cGMP/GC ARC assay can be utilized for the detection and quantification of neutralizing anti-drug antibodies (NAb).**

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