A robust, highly sensitive cell-based assay for potency measurement of the AAV-vectorized optogenetic therapy, GS030-DP, developed to treat retinitis pigmentosa

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PURPOSE

Potency is a critical quality attribute of biological product which is often complex to develop in an industrial compliant format for gene therapy product. However, this is an absolute regulatory requirement to comply with FDA's Current Good Manufacturing Practice (cGMP).

GS030-Drug Product (DP) is an optogenetic AAV-vectorized gene-therapy developed to treat retinal degenerative diseases, such as retinitis pigmentosa, that will enter clinical stage in 2018. GS030-DP consists in an AAV2.7m8 vector and a transgene which encodes the algae red-shifted modified channelrhodopsin, ChrimsonR, fused to the fluorescent protein, tdTomato. CnChR1 also named Chrimson is a microbial opsin isolated from *Chlamydomonas noctigama*. ChrimsonR, the K176R mutant of Chrimson, was optimized with regard to its biophysical properties and was thus selected. ChrimsonR belongs to the large family of channelrhodopsins which are light-gated ion channels.

ChrimsonR-tdTomato essentially localizes to the cell membrane of transduced cells. Upon light stimulation at about 590 nm, ChrimsonR-tdTomato undergoes conformational change and opens, leading to a fast, proton- and cation-driven inward current, triggering plasma membrane depolarization.

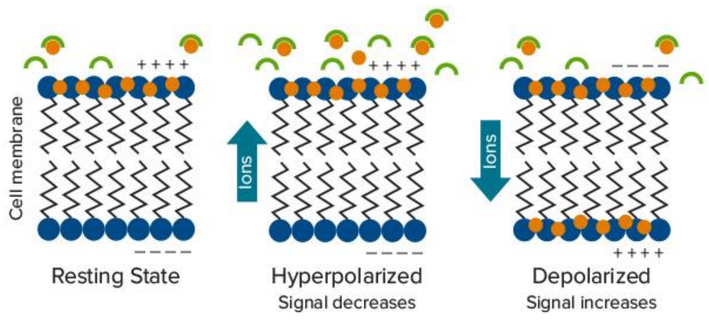
We describe here the development of a specific and highly sensitive potency assay assessing GS030-DP biological function i.e. light-induced depolarization of transduced cells.

METHODS

ASSAY PRINCIPLE:

The FLIPR® Membrane Potential Assay Kit is an homogenous (no wash) fluorescence-based formulation for observation of real-time membrane potential changes associated with ion channel activation and ion transporter proteins. It utilizes a patented indicator dye (oxonol- based) and quencher combination which allows the detection of bidirectional gradient changes, with good assay window and good correlation with manual patch clamp data.

Changes in membrane potential are detected as changes in fluorescence, which are determined by the oxonol dye re-distribution (and consequent un-masking from the quencher) across plasma-membrane following electrical forces: the fluorescent signal increases in intensity during membrane depolarization as the dye follows the positively charged ions inside the cell, thus dissociating from extracellular quencher molecules. During membrane hyperpolarization, fluorescent signal decreases in intensity as dye follows the positively charged ions out of the cell, thus associating with extracellular quencher molecules. Oxonol dye re-distribution reach equilibrium with a time constant of 4 - 8 seconds (*Fairless et al. PLoS One. 2013;8(3)*), thus monitoring membrane voltage changes with a temporal resolution in the second timescale.



EXPERIMENTAL FLOWCHART:

Cells were seeded and infected as described in specific Sections, then analysed with Membrane Potential sensitive dye (MPdye; Molecular Devices) at FLIPR^{TETRA} (ICCD camera). The day of experiment a MPdye Loading Solution was freshly prepared according to manufacturer's instructions, in the indicated Buffer supplemented with All-trans Retinal 5 µM.

Medium was manually removed by plate overthrown and tap on paper towel, then cells were incubated in the dark with 20 µL/w of MPdye Loading solution.

Light stimulation experiments were performed at FLIPR^{TETRA} (ICCD-Camera) using a DUAL Read mode (exc 510-545 nm / em 565-625 nm and exc 610-626 nm / em 646-706 nm) after setting instrument parameters (Excitation % and GateOpen %) in order to obtain basal fluorescence values of around 10,000 RFU (Relative Fluorescence Units). Six minutes fluorescence measurement was performed.

FLIPR^{TETRA} measurements were analysed with Screenworks© software (Molecular Devices, Version 4.0.0.30). Percentage Fluorescence changes (ΔF%) were obtained applying "Response over Baseline", "Subtract Background" and "Show as percentage" corrections, then exporting (Maximum – Minimum) response value. Mean and standard deviation of replicates were calculated with Microsoft Excel software then used to fit sigmoidal dose-response curves (variable slope; Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope)), using GraphPad PRISM software.

To evaluate assay signal dynamic range and data variation associated with the signal measurements, Z' factor value was determined for each assay plate according to the following formula and therefore used for assay quality assessment:

$$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

where means (µ) and standard deviations (σ) of both the positive (p) and negative (n) controls (μ_p, σ_p, and μ_n, σ_n) are used. Positive (or MAX) controls correspond to maximal EC100 concentration of GS030-DP (1^E+5 MOIs), while negative (or MIN) controls correspond to not infected cells. The obtained Z' value is interpreted as following (*Zhang et al., Biomol Screen. 1999;4(2):67-73*):

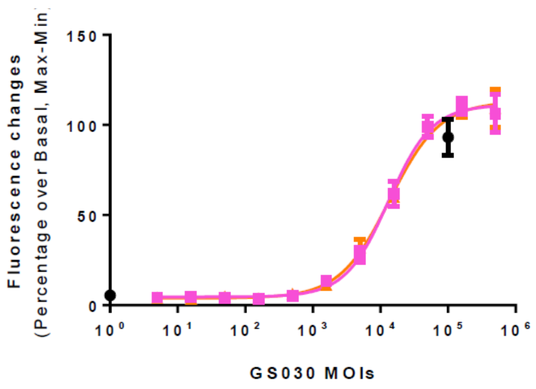
Z-Factor	Interpretation
1.0	Ideal Z-factor can never exceed 1
Between 0.5 and 1.0	An excellent assay.
Between 0 and 0.5	A marginal assay
Less than 0	There is too much overlap between the positive and negative controls for the assay to be useful

2. RESULTS - CLINICAL BATCH ROUTINE TESTING (R&D GRADE)

The clinical batch has been tested versus the Reference batch.

Potency was expressed as EC50 percentage variation, corresponding to the following formula:

[EC50 REF/EC50 SAMPLE]*100



Z' wells (ref. batch)

	mean	st Dev	CV
Min	5.39	2.04	38%
Max (1E05 MOI)	93.14	9.96	11%
Z'	0.590		

	Titer	EC50	Log EC50	Top
Reference batch	7.84E+12 vg/mL	13709	4.14	113
Clinical batch (sample)	4.65E+12 vg/mlL	13182	4.12	111

EC50 ref/ EC50 sample*100
=
104

The present test showed: - good assay performance, both in term of Z' factor value (higher than 0.5) and
- biological activity of the GMP tested batch compared to the Reference batch, based on EC50 value comparison.

CONCLUSION

The results of this assay development can be summarized as following:

- Multi-Plate tests run in 3 different days on the generated GS030-DP potency assay showed very good robustness and repeatability, with good intra-plate, intra-day and inter-day precision, both as un-sensibility to small deliberate variations of method procedure, and good sensitivity to deliberate small variations of the reference batch concentration.

- The potency of different batches of GS030-DP product analyzed in 3 different experimental days was efficiently discriminated by the generated potency assay, showing very good inter-day precision and assay robustness.

- The evaluation of the first clinical batch showed a similar potency to the reference batch.

→ Next step will consist to validate the analytical method and accumulate data on clinical batches & stability studies.

→ This robust potency assay will support the next steps of the GS030 gene therapy, especially for batch release and stability evaluation. This will also allow to comply with regulatory requirements in order to move to pivotal studies and ultimately to BLA/MA.

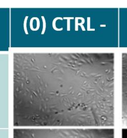
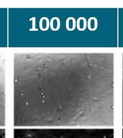
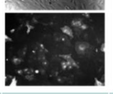
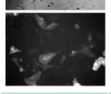
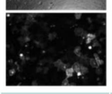
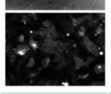
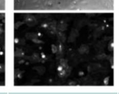
1. RESULTS - DEVELOPMENT

1.1-Method development

In order to improve GS030-DP infection efficiency several protocol optimization have been set:

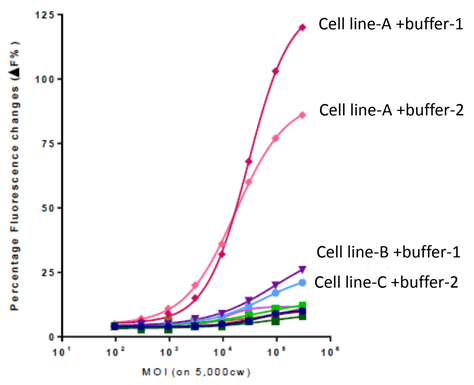
- Cell seeding density
- Cell lines permissiveness: 4 cell lines evaluated
- Transduction step
- and Infection time
- Multiplicity Of Infection (MOI) range

The permissiveness of the cells was evaluated using the fluorescence measurement of the expressed tdTomato gene:

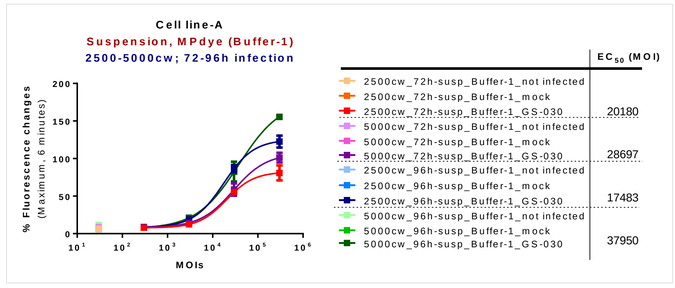
MOI	(0) CTRL -	300	1000	3000	10 000	30 000	100 000	300 000
Phase								
Red fluo-rescence								
% red cells	0%	10%-20%		50%			100%	100%

Microscopic observation 72H after GS030 transduction (tdTomato fluorescence)

Then, the protocol parameters were evaluated with ChrimsonR protein activation:



Comparison of GS030-DP transduction efficiency on cell lines

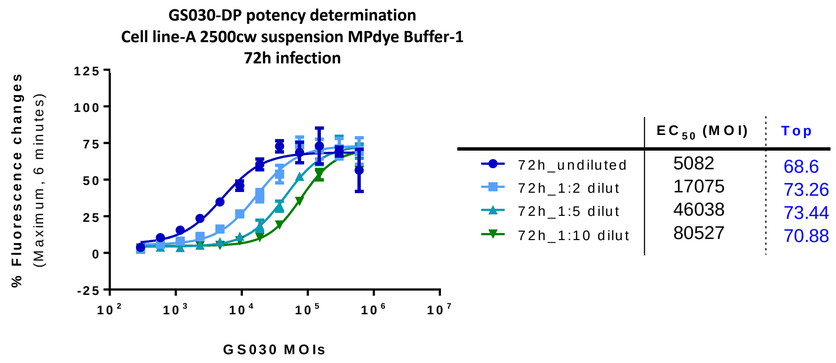


Optimization of cell seeding and infection time

The following conditions have been chosen as optimal for GS030-DP potency assay :

- Cell line-A
- Cell density: 2500c/w
- Infection protocol: suspension by spinoculation
- Infection time: 72h
- Assay buffer: buffer-1

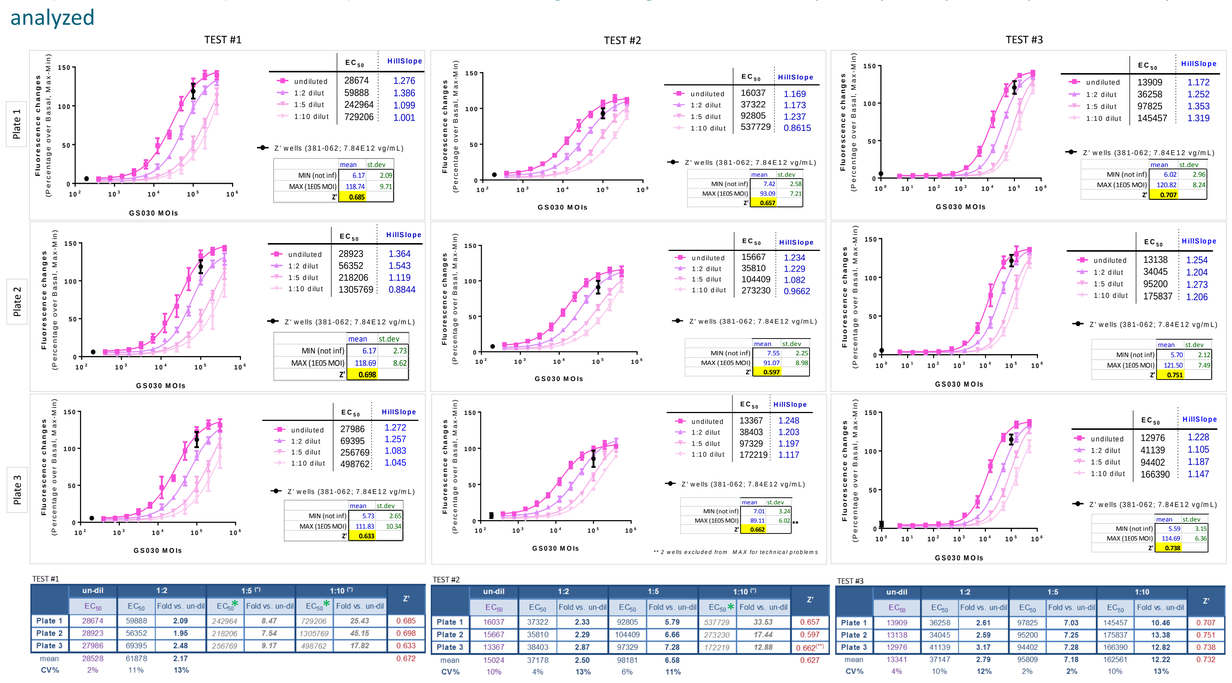
In order to preliminarily evaluate if the assay is able to give dose-response curve and to detect minor changes in batch potency, a full titration of GS030-DP using 11 MOI conditions have been tested both with undiluted or 1:2, 1:5, 1:10 diluted batch.



1.2-Assay robustness and Multi-Plate test

❑ Different concentration ranges and dilution steps were used for the 11-points dose response, in order to identify the optimal condition that would enable a good definition of both lower and upper plateau levels of batches with different potencies.

In order to simulate minor changes in batch activity, each concentration of the 11- points dose-response prepared with undiluted GS030-DP was further diluted to obtain the 1:2, 1:5 or 1:10 diluted 11-points dose-responses. Moreover, assay window and signal robustness was evaluated testing the response of MAXIMUM (EC100 concentration of GS030-DP: 1^E+05 MOI) and MINIMUM (not infected) wells and calculating resulting Z' value. Three plates per day in 3 experimental days were analyzed



TEST #1

	un-dil EC50	1:2 EC50	1:2 Fold vs. un-dil	1:5 (*) EC50*	1:5 Fold vs. un-dil	1:10 (*) EC50*	1:10 Fold vs. un-dil	Z'
Plate 1	28674	59888	2.09	242964	8.47	729206	25.43	0.685
Plate 2	28923	56352	1.95	218206	7.54	1305769	45.15	0.698
Plate 3	27986	69395	2.48	256769	9.17	498762	17.82	0.633
mean	28528	61878	2.17					0.672
CV%	2%	11%	13%					

TEST #2

	un-dil EC50	1:2 EC50	1:2 Fold vs. un-dil	1:5 EC50	1:5 Fold vs. un-dil	1:10 (*) EC50*	1:10 Fold vs. un-dil	Z'
Plate 1	16037	37322	2.33	92805	5.79	537729	33.53	0.657
Plate 2	15667	35810	2.29	104409	6.66	273230	17.44	0.597
Plate 3	13367	38403	2.87	97329	7.28	172219	12.88	0.662(**)
mean	15024	37178	2.50	98181	6.58			0.627
CV%	10%	4%	13%	6%	11%			

TEST #3

	un-dil EC50	1:2 EC50	1:2 Fold vs. un-dil	1:5 EC50	1:5 Fold vs. un-dil	1:10 EC50	1:10 Fold vs. un-dil	Z'
Plate 1	13909	36258	2.61	97825	7.03	145457	10.46	0.707
Plate 2	13138	34045	2.59	95200	7.25	175837	13.38	0.751
Plate 3	12976	41139	3.17	94402	7.28	166390	12.82	0.738
mean	13341	37147	2.79	95809	7.18	162561	12.22	0.732
CV%	4%	10%	12%	2%	2%	10%	13%	

Dose response curve didn't reach the high plateau, thus EC50 value shouldn't be considered reliable
"Fold vs. un-dil" = EC50 (1:2 or 1:5 or 1:10 diluted stock) / EC50 (un-diluted stock)

❑ Small method variations were introduced in the 3 different experimental days:

- Pre-culture confluency: 80 to 95% by visual inspection
- Days of pre-culture: 4 or 5
- Trypsin removal before cell seeding & infection: yes or no
- Plate spin after viral particle addition: 20min or 10min + 10min (800rpm)
- FLIPR-TETRA reader: #2 or #4

Showing that assay performance was not affected, both in terms of EC50 and Z' factor values.

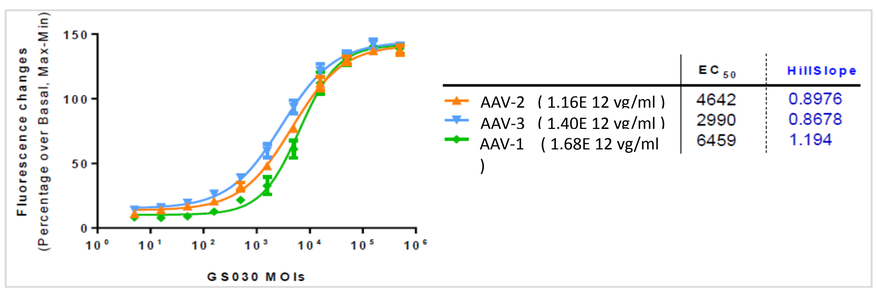
3-Batch-to-batch variability tests

In order to determine the ability of the so far generated potency assay to discriminate between different batches of GS030-DP product, the best experimental conditions were used to run batch-to-batch variability tests.

3 batches were analyzed on 3 different days :

Batch	Production protocol	Scale	Viral genome titer (VG/ml)	Infectious titer (TCID50/ml)
AAV-1	Tri transfection	Small	1.68^E+12	2.96^E+10
AAV-2	Baculovirus	Medium	1.16^E+12	9.37^E+10
AAV-3	Baculovirus	Large	1.40^E+12	7.74^E+10

Results from 1-day experiment is presented below:



=> EC50 results of the 3 batches are close.