Is recombinant Insulin a factor to consider for viral production?

Viral systems are commonly used in both R&D and therapeutic applications for the delivery of transgenes in cell & gene therapies and vaccine production. This area is in expansion and creates increasing needs for production scale-up, optimized processes and increased yields. For safety reasons, many choose an animal component-free, CD cell culture media with added metabolites, such as recombinant insulin.

From previously published data, significant improvements have been shown using insulin for viral production. In a case study on HEK 293 for influenza HA protein[1)], a 4-fold increase for viable cell growth and 60% increase in titre were observed in CD media with 10 mg/L insulin. Using HEK 293 for HIV-1 Gag Virus-Like-Particles (VLPs) in CD medium supplemented with 20 mg/L insulin[2)], VLP production in supplemented medium was increased 1.9-fold, proportionally to the improvement in cell growth.

Therefore our hypothesis was, that recombinant insulin has a positive effect on the production titre for Lentivirus (LV) and Adeno-Associated Virus (AAV) in HEK 293.
Objective: Study the effect of insulin concentration and addition time on cell growth and viral expression in HEK293 cells.

Materials and methods:

- **Adeno-Associated Virus (AAV)** serotypes **AAV2, AAV5 and AAV8**; 3 plasmid packing system
- **Lentivirus (LV)**; 4 plasmid (GFP) packing system
- Cell line: Suspension adapted Wayne293™ (HEK 293)
- Reactors: 125 ml shake flask reactors, 30 ml working volume
- Media: Wayne293™ CD Transfection medium (packaging and growth) + FEED03 Medium
- Additive: Recombinant Insulin Human AF (Novo Nordisk Pharmatech A/S) to the basal medium
- Transfection reagent: PEI
- Virus particles collected 72h after transfection.
- Cell density and viability sampling time: each day
- AAV Titre: The AAV titre in supernatant was calculated by detecting the ITR gene using real-time PCR
- LV Titre: HT1080 cells infected with LV. GFP gene expression was detected after 72 hours of infection using FACS.

Experimental conditions:

Table 1. Experimental materials and conditions for AAV and LV

Experiment Runs #:		20 runs	
Start date:	2021.08.09	**Completion:**	2021.08.23
Suspension Cell line:		Wayne 293 cell line (host) and HT1080 (LV)	
Researcher(s):	QUACELL R&D dept.	**Packaging Medium:**	WAYNE293 TM Transfection Medium (serum free)
Reactor:	125ml shake flask	**Growth Medium:**	WAYNE293 TM Transfection Medium (serum free)
Working Volume:	30ml	**Feed Medium:**	FEED03 Medium (serum free)
Inoculation Density:	5E+05 cells/ml		
Condition:		37°C, 5%CO2, 100rpm, Humidity 80%	
Feeding strategies:		Glucose: Add 6g/L if glucose lower than 3g/L Feed 03: Added 2 hours before transfection	
Harvest conditions:		Collect Virus particles containing supernatant at 72 hours after transfection	
Sampling Time:		Everyday	

Lentivirus:
The four plasmid system containing GFP tags was transferred into the host cell to produce LV particles. After 48 hours the LV particles were collected and HT1080 cells were infected with LV. 72 h post infection, the positive expression rate of GFP in HT1080 cells was observed using FACS and the infection titre (Tu/mL) of LV virus in the supernatant was calculated.

Seed	Transfection	Harvest	Infection	Titre detected
Seed Wayne293 cells in shake flask	Transfection Wayne293 cell line with LV four plasmid system	After 48h, harvest the LV particles by collecting the supernatant	Infection HT1080 cells with supernatant	After 72h, target gene (GFP) detected by FACS method (Tu/ml)

AAV:
The three plasmid system was transferred into the host cell to produce AAV particles. After 72 hours collecting the AAV particles (cell lysis), DNA extraction kits were used to extract viral DNA. The content of ITR gene in the DNA was detected by qPCR method, and the titre (VG/mL) of AAV virus in the supernatant was calculated.

Seed	Transfection	Harvest	DNA extraction	Titre detected
Seed Wayne293 cells in shake flask	Transfection Wayne293 cell line with AAV three plasmid system	Harvest the AAV particles by lysing Wayne293 cells	After 72h, extract viral DNA	Target gene (ITR) detected by qPCR method (VG/ml)

We used a 3x2 independent factor design.

Table 2. Experimental Run design

Run No.	Insulin add time	Insulin concentration (mg/L)
1, 2	Control	0
3, 4	A: At cell inoculation	5
5, 6		10
7, 8		20
9, 10	B: 2 hours before transfection	5
11, 12		10
13, 14		20
15, 16	C: 4 hours after transfection	5
17, 18		10
19, 20		20

We investigated the following parameters:
(1) The effect of insulin concentration on cell growth
(2) The effect of insulin concentration on the expression of virus package
(3) The effect of insulin addition time on cell growth
(4) The effect of insulin addition time on the expression of virus package

Results

Figure 1. Cell viability AAV-2, AAV-5, AAV-8

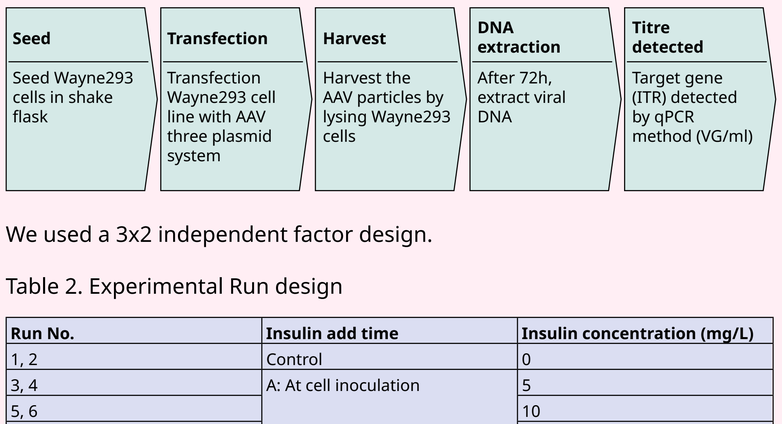


Figure 2. Cell density AAV-2, AAV-5, AAV-8

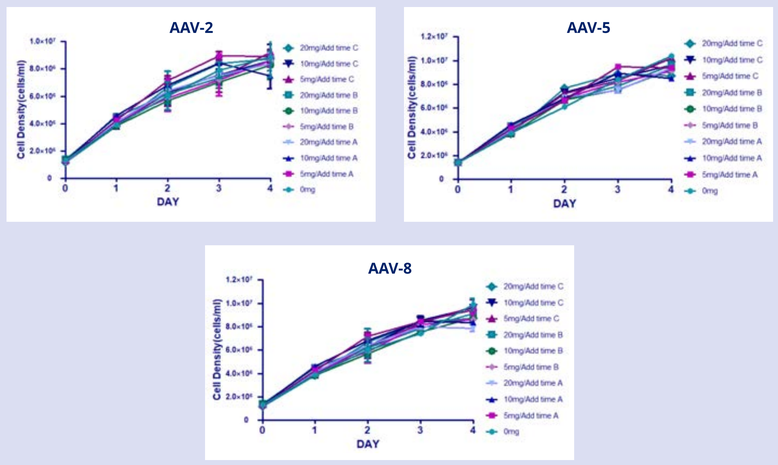


Figure 3. Titre AAV-2, AAV-5, AAV-8

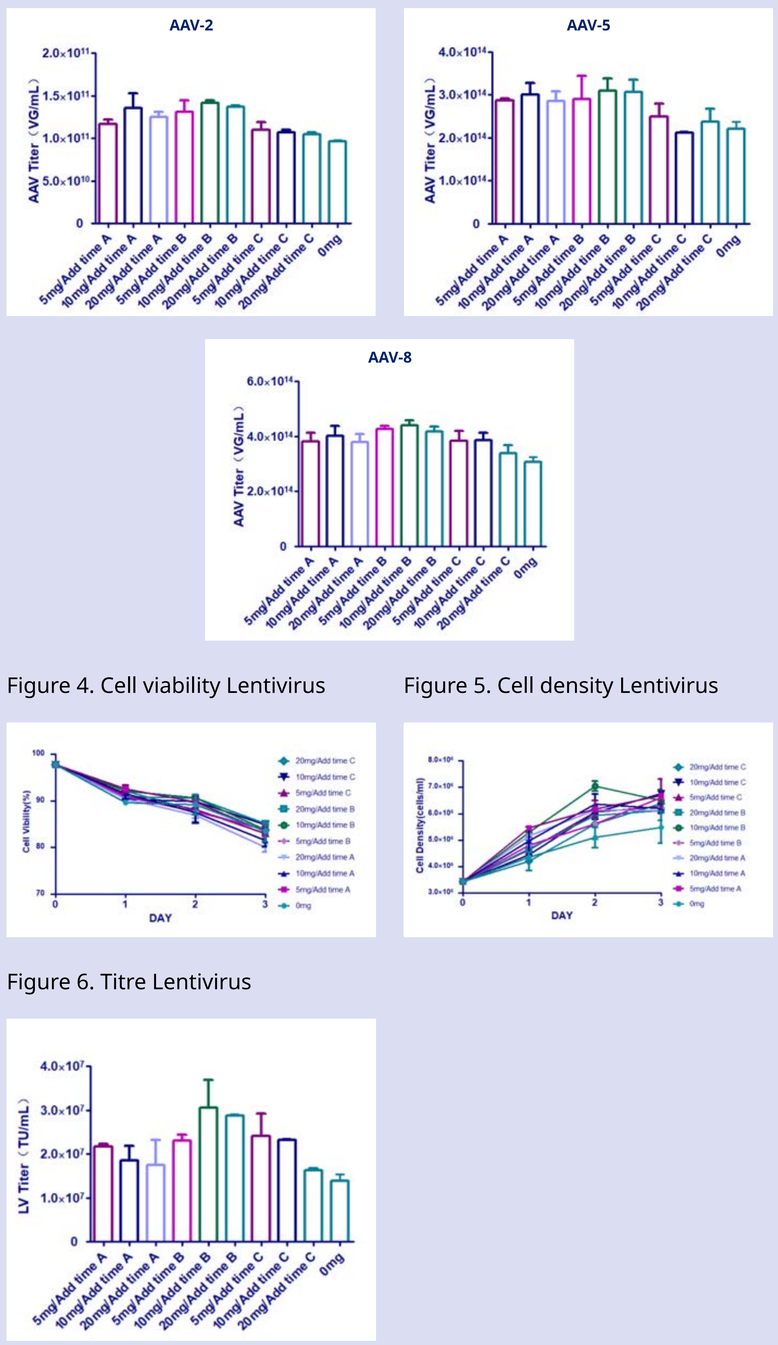


Figure 4. Cell viability Lentivirus

Figure 5. Cell density Lentivirus

Figure 6. Titre Lentivirus

For all results above, the two parallel raw data were processed into Graphpad Prism. The error bars were calculated from the mean.

Discussion - AAV
(1) Insulin concentration on cell growth
- Cell viability and density: R-Insulin showed no significant effect in cell viability or cell density maintenance compared with the control group, also no difference between the three serotypes.

(2) Insulin concentration on AAV titre

	Titre with NO Insulin	Highest titre WITH Insulin	Insulin Concentration	Trend
AAV-2	9.81E+10 VG/mL	1.53E+11 VG/mL	10 mg/ml	+56%
AAV-5	2.04E+14 VG/mL	3.44E+14 VG/mL	5 mg/ml	+68%
AAV-8	2.88E+14 VG/mL	4.37E+14 VG/mL	5 mg/ml	+51%

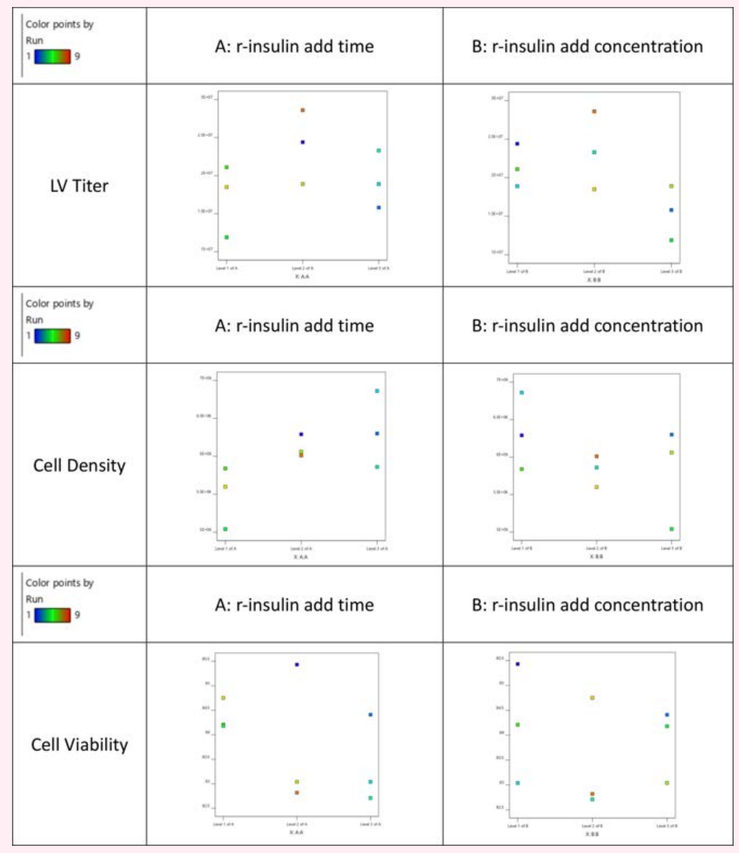
(3) Insulin addition time on cell growth
- Cell viability and density: The different addition times showed no significant difference in cell viability or cell density maintenance compared with the control group, also no difference between the three serotypes.

(4) Insulin addition time on AAV titre
Different adding times had a certain influence on the titre level of AAV. Adding insulin at inoculation, AAV-2 titre increased by 35% and reached 1.53E+11 VG/mL, compared with 1.10E+11 VG/mL when adding at 4 hours post transfection. We observed the same for serotypes AAV-5 and AAV-8, where adding insulin at inoculation and 2 hours before transfection also increased titre by 68% (AAV-5) and 51% (AAV-8) compared with 4 hours after transfection.

Conclusion AAV
In this experiment, adding 5-10mg/L insulin at the time of inoculation or 2 hours before transfection can improve AAV titre in serum-free suspension system, but does not affect the maintenance of cell density or cell viability

DOE for multi-factor analysis (Design Expert v. 12.0) for AAV2, AAV5, AAV8 is not shown. Figures and full report available on request.

Figure 8. DOE for multi-factor analysis (Design Expert v. 12.0) – LV



Discussion - Lentivirus
(1) Insulin concentration on cell growth
- Cell viability: The addition of 5mg/L, 10mg/L and 20mg/L insulin showed no significant difference in cell viability maintenance compared with the control group.
- Cell density:

72h post transfection	Cell density with NO Insulin	Cell density WITH Insulin	Insulin Concentration	Trend
	4.88E+06 cells/mL	6.86E+06 cells/mL	5 mg/ml	+40%

The results showed that insulin had a certain effect on the increase of cell density during virus packaging. However, when insulin concentration was increased to 10mg/L and 20mg/L, cell density did not increase with insulin concentration.

(2) Insulin concentration on LV titre

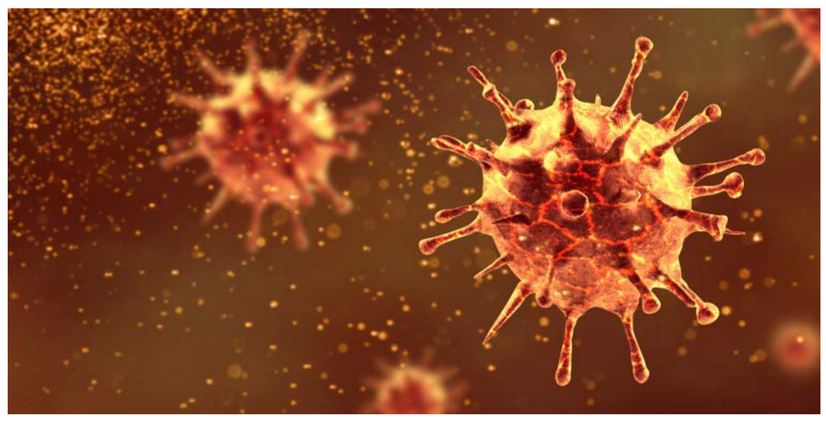
LV	Titre with NO Insulin	Highest titre WITH Insulin	Insulin Concentration	Trend
	1.39E+07 TU/mL	3.06E+07 TU/mL	10 mg/ml	+120%

Continuing to increase insulin concentration to 20mg/L did not increase virus production.

(3) Insulin addition time on cell growth
- Cell viability: Different time of insulin addition had no significant effect on cell viability maintenance.
- Cell density: When insulin was added 4 hours after transfection, the cell density could reach 6.86E+06 cells/mL 72 hours after transfection. The increase in cell density was 17% and 9%, respectively, compared with the addition time at inoculation (5.84E+06 cells/mL) and 2 h before transfection (6.29E+06 cells/mL).

(4) Insulin addition time on LV titre
Different adding times had a certain influence on the expression level of virus. Adding insulin 2 hours before transfection, LV titre reached 2.86E+07 TU/mL, compared with adding at cell inoculation (2.11E+07 TU/mL) and adding 4 hours after transfection (2.33E+07 TU/mL). LV titre increased by 35% and 22%, respectively.

Conclusion Lentivirus
In this experiment, Adding 10mg/L insulin 2 hours before transfection can improve cell density and lentivirus titre in serum-free suspension system, but does not affect the maintenance of cell viability.



References:
1. "Why use recombinant Insulin in DoE for your media development?" Novo Nordisk Pharmatech A/S, Cell Series UK 2020 / "Effect of Insulin on Cell Growth and Virus Production", A. Manceur et al., NRC Canada, 2016.
2. "Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium." L. Cervera et al., Journal of Biotechnology 166 (2013) 152–165.

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Novo Nordisk Pharmatech A/S
Koebenhavnsvej 216, DK-4600 Koege
Phone: +45 5667 1000
nnprinfo@novonordisk.com
novonordiskpharmatech.com

Novo Nordisk Pharmatech A/S | novo nordisk®