

AAV production

Production of AAV using the AAV-MAX system in a 3 L stirred-tank bioreactor

Introduction

The Gibco™ AAV-MAX Helper-Free AAV Production System is an adeno-associated virus (AAV) production system developed for scalable, high-titer AAV production. The AAV-MAX system includes a HEK293F-derived clonal suspension cell line adapted to a chemically defined medium; transfection reagent; transfection booster; and a production enhancer. All components of the system are animal origin-free.

In this application note, we provide guidance for using the AAV-MAX system to produce AAV in the Thermo Scientific™ HyPerforma™ Glass Bioreactor. The bioreactor control and process parameters presented here were optimized for AAV production. The inoculation strategy, agitation, pH control, and complexation parameters were evaluated using either AAV serotype 2 or 6. By optimizing the bioreactor process parameters, AAV production in 3 L HyPerforma bioreactors provided growth kinetics and metabolite profiles that were comparable to those achieved in shake flask production. Bioreactor production also generated reproducible AAV2 and AAV6 titers that were equivalent or superior to those obtained in shake flask production.

Materials

- 3 L HyPerforma Glass Bioreactor, 120 V, heat only
- Thermo Scientific™ HyPerforma™ G3Lab Controller for HyPerforma Glass Bioreactor, non-GMP
- Gibco™ Viral Production Cells 2.0
- Gibco™ Viral Production Medium
- Gibco™ AAV-MAX Transfection Kit
 - Gibco™ AAV-MAX Transfection Reagent
 - Gibco™ AAV-MAX Transfection Booster
 - Gibco™ AAV-MAX Enhancer
- Gibco™ Viral-Plex™ Complexation Buffer
- Gibco™ GlutaMAX™ Supplement

Methods

Bioreactor preparation, cell expansion, and inoculation Expand cells (Day –4)

For cell expansion, Viral Production Cells (VPC) 2.0 were thawed in Viral Production Medium (VPM) supplemented with 4 mM GlutaMAX Supplement. The cells were thawed and maintained according to the AAV-MAX Helper-Free Production System User Guide (Pub No. MAN0019619). Three days prior to inoculating each bioreactor, a shake flask was inoculated to a final density of 0.6×10^6 viable cells per mL (VC/mL) in a total volume of 1 L and grown to a density of $4\text{--}6 \times 10^6$ VC/mL. This culture yielded the 2.7×10^9 viable cells required to inoculate one stirred-tank bioreactor to a final density of 1.5×10^6 VC/mL in a total volume of 1.8 L.

Prepare bioreactor (Day –3 or Day –2)

The 3 L HyPerforma Glass Bioreactor was prepared either two or three days prior to transfection depending on which inoculation strategy was used. The bioreactor was assembled, and the pH and dissolved oxygen (DO) probes were calibrated and attached to the bioreactor. The bioreactor was then sterilized in an autoclave. After sterilization, the bioreactor was cooled to room temperature.

Inoculate bioreactor (Day –2 or Day –1)

The parameters shown in Table 1 were applied to produce AAV in 3 L HyPerforma glass stirred-tank bioreactors.

Table 1. Recommended bioreactor settings for AAV production.

Parameter	Setting
Vessel	3 L HyPerforma Glass Bioreactor
Controller	HyPerforma G3Lab Controller
Sparge	L-shaped drilled hole macrosparge
Impellers	1 x Rushton (bottom); 1 x 3 pitched blade (top)
Impeller diameter	55 mm
Impeller power number	1.4
Agitation	230 rpm; P/V: 20 W/m ³ ; tip speed: 0.66 m/s
Working volume	2 L
Temperature	37 ± 0.5°C
Overlay gas	Air (0.05 L/m)
Dissolved O ₂	40% (controlled with O ₂ through the sparge)
pH	7.0 ± 0.2 (controlled with CO ₂ through the sparge)

Two inoculation strategies (Strategy 1 and Strategy 2) were evaluated. For inoculation by Strategy 1, VPC 2.0 were grown to a density of $3.0 \pm 0.5 \times 10^6$ VC/mL in the bioreactor. AAV-MAX Enhancer was then added, and the cells were transfected without the addition of fresh VPM. For Strategy 2, VPC 2.0 were grown to a density of $6.0 \pm 0.5 \times 10^6$ VC/mL and diluted to 3.0×10^6 VC/mL with fresh VPM prior to the addition of AAV-MAX Enhancer and transfection. While AAV is typically harvested 72 hours posttransfection, providing VPC 2.0 with fresh medium according to Strategy 2 may be beneficial when a later harvest time is indicated. This will minimize the potential for nutrient depletion during the run. For both strategies, the stir speed and temperature were set to 20 W/m³ and 37°C, respectively. The overlay airflow was set to 0.05 L/min, while DO was set to 40% and controlled with O₂ on demand through the sparge.

Inoculation Strategy 1

One day before transfection (Day -1), 1 L of VPM was added to the bioreactor and equilibrated to 37°C prior to inoculation. The bioreactor was inoculated by adding VPC 2.0 to a final density of 1.5×10^6 VC/mL in a total volume of 1.8 L. The cells were then grown to the target density of $3.0 \pm 0.5 \times 10^6$ VC/mL within 20–24 hours of inoculation.

Inoculation Strategy 2

Two days prior to transfection (Day -2), the bioreactor was inoculated by adding VPC 2.0 to a final density of 1.5×10^6 VC/mL in a total volume of 1 L. The cells were grown to the target density of 6×10^6 VC/mL on the day of transfection (Day 0).

Postinoculation parameters

The pH was checked off-line with a blood gas analyzer after inoculation, and a one-point calibration was performed. After calibration, the pH was set to 7.0 ± 0.2 and controlled on demand with CO₂ through the sparge. For all experiments,

Table 2. Recommended reagent volumes for a 2 L transfection.

Reagent	Volume	Percentage of culture volume
Culture volume to transfect	1.8 L	100%
Volume of plasmid DNA*	2.7 mL	0.15%
Viral-Plex Complexation Buffer	180 mL	10%
AAV-MAX Transfection Reagent	10.8 mL	0.6%
AAV-MAX Transfection Booster	5.4 mL	0.3%
AAV-MAX Enhancer	18 mL	1.0%
Final culture volume	2 L	112.05%

* Assuming a stock plasmid DNA concentration of 1 mg/mL.

control cultures were set up at a 30 mL production scale in vented, non-baffled 125 mL shake flasks as described in the AAV-MAX user guide.

Transfection and harvest

Check cells (Day 0)

On the day of transfection, the viable cell density (VCD) and viability of the cultures were assessed. A final VCD of $3.0 \pm 0.5 \times 10^6$ VC/mL and $\geq 95\%$ viability is recommended before proceeding with transfection.

Dilute culture (Day 0)

The culture obtained by Strategy 2 was diluted on Day 0 with fresh VPM to a final target VCD of $3.0 \pm 0.5 \times 10^6$ VC/mL in a final volume of 1.8 L prior to the addition of AAV-MAX Enhancer. The culture obtained by Strategy 1 was not diluted with fresh VPM.

Add enhancer (Day 0)

Thirty minutes prior to transfection, the size 16 tubing on the transfer cap of a 250 mL Thermo Scientific™ Nalgene™ PETG bottle containing AAV-MAX Enhancer was sterile-welded to equally sized tubing on the bioreactor. After welding, the enhancer was transferred to the bioreactor using a peristaltic pump set to 70 mL/min.

Transfect (Day 0)

To prepare the transfection complex, cold Viral-Plex Complexation Buffer was added to a 250 mL Nalgene PETG bottle. A mixture of transfer plasmid DNA, Rep/Cap plasmid DNA, and helper plasmid DNA in a 1:3:1 mass ratio was added to the complexation buffer. The bottle was gently swirled to mix the contents, and then incubated at room temperature for 10 minutes.

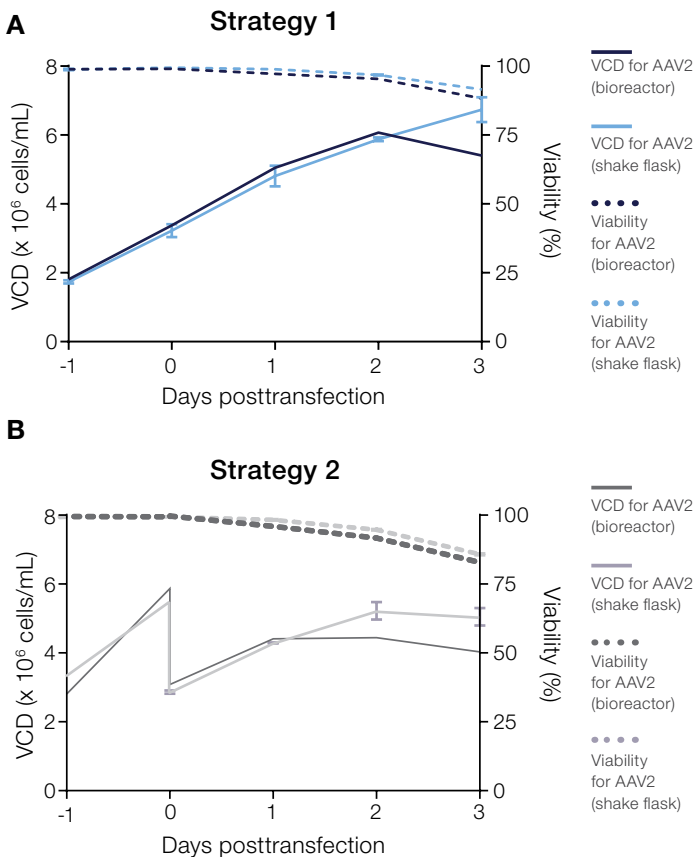
AAV-MAX Transfection Booster was transferred to a 50 mL polypropylene conical tube, followed by the AAV-MAX Transfection Reagent. The conical tube was gently swirled to mix the contents, then incubated at room temperature for 10 minutes.

After the 10-minute incubation period, the solution containing AAV-MAX Transfection Reagent and AAV-MAX Transfection Booster was transferred to the bottle containing the diluted plasmid DNA. The bottle was gently swirled to mix the contents, and then incubated at room temperature for 10 minutes. During incubation, a transfer cap was placed on the bottle, and the bottle was connected to the bioreactor with sterile-welded tubing. After incubation was complete, the solution was transferred to the bioreactor using a peristaltic pump set to 70 mL/min.

Each culture was sampled daily following transfection. The density, viability, and size of the cells were determined using a Vi-CELL™ cell viability analyzer (Beckman Coulter). The pH, O₂, and CO₂ levels were measured on a Stat Profile Prime™ CCS Analyzer (Nova Biomedical). Glucose, glutamine, glutamate, lactate, and ammonia levels were measured on a Cedex™ Bio HT bioanalyzer (Roche Diagnostics).

Harvest (Day 3)

Prior to harvest, 0.9 mL of unclarified culture was harvested and frozen at -80°C for titer determination by real-time quantitative PCR (qPCR).



Quantitation of viral genome titers by qPCR

The frozen, unclarified harvests were thawed on ice for 30 minutes and lysed by adding 0.1 mL of 10X AAV-MAX lysis buffer. AAV genome titers were determined as described in the AAV-MAX user guide. Prior to qPCR analysis, the crude lysates were subjected to DNase I and Exo I treatment to remove non-encapsidated DNA. This was followed by proteinase K treatment to release the encapsidated DNA from the AAV particles. qPCR was performed using primers and probes that targeted the gene for green fluorescent protein (GFP), which was flanked by the inverted terminal repeat (ITR) sequences of the packaged viral DNA. The AAV genome titers were determined based on a standard curve for linearized GFP-containing plasmid DNA. Refer to the AAV-MAX user guide for additional details.

Results

Similar growth profiles were observed in the bioreactors and the corresponding shake flask controls after inoculation by both strategies (Figures 1A and 1B). Each inoculation strategy also yielded similar AAV2 genome titers (Figure 1C). The titers in the bioreactors trended higher than those in the corresponding shake flask controls. Strategies 1 and 2 yielded comparable results as indicated by the cell growth, viability, and titer data. Based on the streamlined process and scheduling considerations, Strategy 1 was chosen for future experiments and is outlined in the protocol provided in the Appendix.

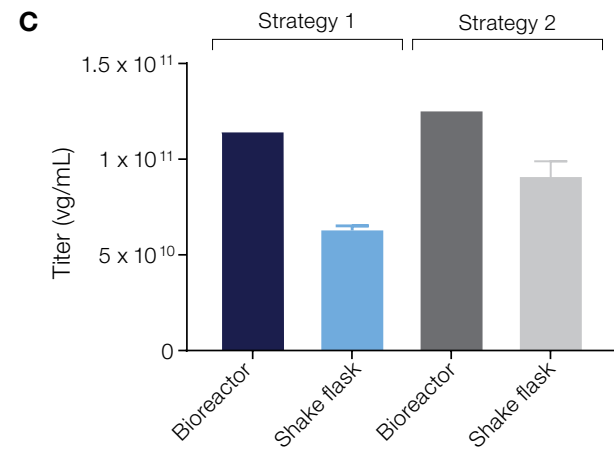


Figure 1. Comparison of two inoculation strategies for AAV2 production. (A) Viable cell density (VCD) and viability of a culture grown in Gibco Viral Production Medium (VPM) for AAV2 production following inoculation Strategy 1 in a bioreactor (n = 1) and in control shake flasks (n = 2). The solid and dotted lines represent VCD and viability, respectively. **(B)** VCD and viability of a culture grown for AAV2 production following inoculation Strategy 2 in a bioreactor (n = 1) and in control shake flasks (n = 2). **(C)** AAV2 genome titers in bioreactors and control shake flasks following inoculation Strategy 1 or Strategy 2.

Following selection of inoculation Strategy 1, growth kinetics, metabolic profiles, and titers were characterized for AAV2 and AAV6 production. Similar cell densities, viabilities, and diameters were observed in the bioreactor and shake flask control cultures (Figures 2A and 2B). Similar profiles for lactate production, ammonia production, and glucose depletion were observed in the bioreactors and shake flask control cultures (Figures 2D–F). The AAV2 and AAV6 genome titers were both higher in the bioreactor cultures than they were in the corresponding shake flask controls. However, the average titers were within 25% of 1×10^{11} vg/mL. Applying the AAV-MAX system in bioreactors using the processes described here reproducibly yielded 1×10^{11} vg/mL of AAV2 and AAV6 particles that contained the GFP gene.

Conclusions

The data collected in this study demonstrate the scalability of the AAV-MAX Helper-Free AAV Production System. AAV2 and AAV6 production was evaluated in 125 mL shake flasks and the 3 L HyPerforma Glass Bioreactor. The selected bioreactor process parameters supported cell growth, and the AAV genome titers obtained using the bioreactor process exceeded the titers obtained by shake flask production. Two inoculation strategies were tested in the study. The selected strategy resulted in comparable performance, streamlined the process, and minimized production time. The results demonstrate successful scale-up of AAV production in 3 L bioreactors with the AAV-MAX system, and we have established suitable parameters for further scale-up.

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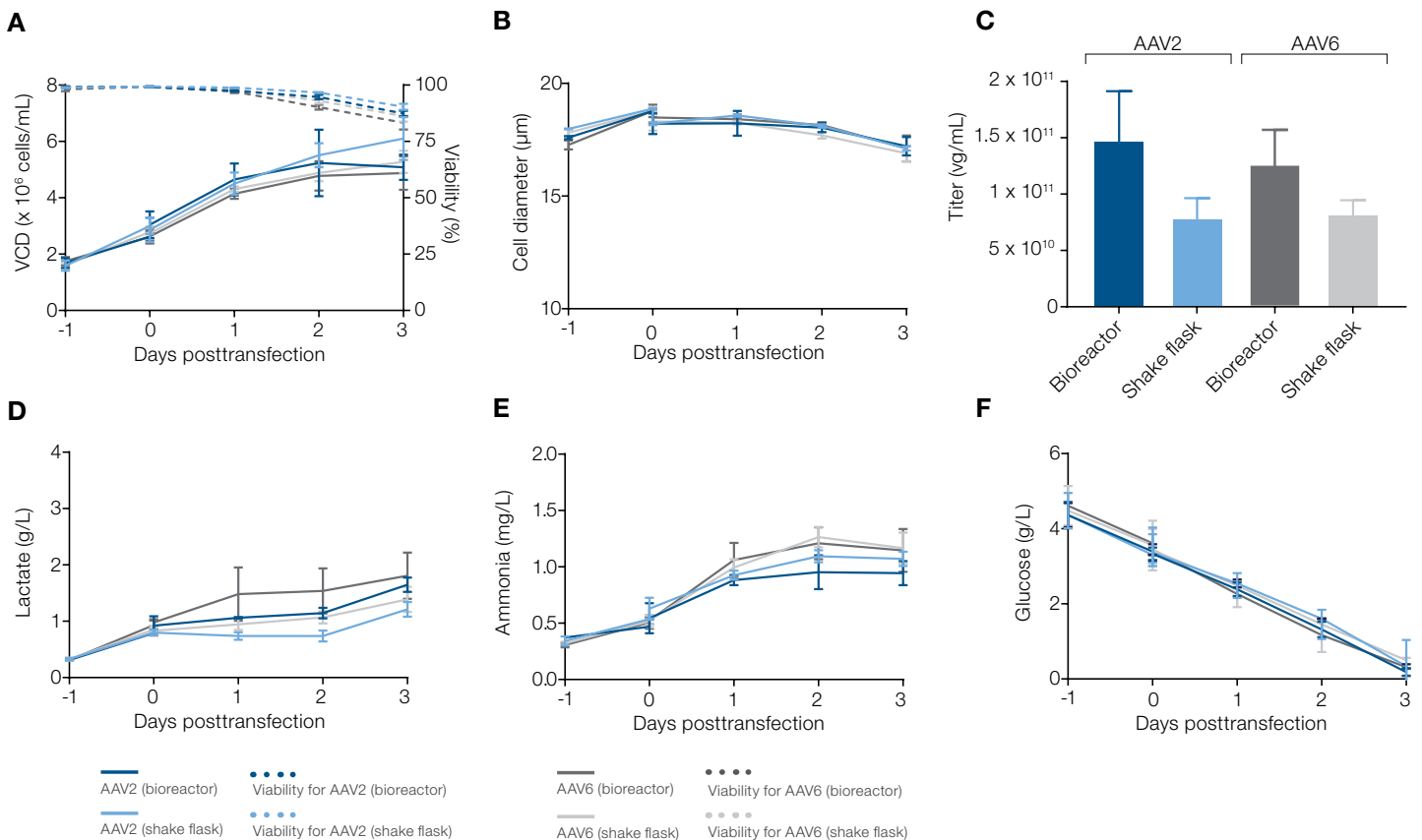


Figure 2. Growth, titers, and metabolic profiles for AAV2 and AAV6 production in bioreactors and shake flasks. (A) Solid lines represent VCD, and dotted lines represent cell viability. Data collected during AAV2 production in a bioreactor are shown in dark blue ($n = 2$). The dark gray lines represent data collected during AAV6 production in a bioreactor ($n = 4$). The light blue and light gray lines represent data collected during AAV2 ($n = 5$) and AAV6 ($n = 4$) production in control shake flasks, respectively. AAV2 production and AAV6 production in shake flasks were each evaluated in 2 independent experiments. (B) Cell diameter, (C) AAV titer, and concentrations of (D) lactate, (E) ammonia, and (F) glucose were also measured.

Appendix

3 L bioreactor protocol

1. Subculture and expand VPC 2.0 until they reach a density of $4\text{--}6 \times 10^6$ viable cells per mL (VC/mL) in a culture volume that will yield at least 2.7×10^9 cells to inoculate 1 bioreactor. This is equivalent to 1.5×10^6 VC/mL in the 1.8 L inoculated culture.
Note: Allow thawed cells to recover for at least 3 passages before transfection.
2. Calibrate the DO and pH probes and install them on the assembled bioreactor.
3. Sterilize the bioreactor and cool it to room temperature.
4. Add 1 L VPM to the bioreactor. Set and enable agitation, headspace gassing, and temperature control as outlined in Table 1. Allow the medium to equilibrate to the temperature set point before seeding the cells.
5. Inoculate the bioreactor by adding VPC 2.0 from step 1 to a final density of 1.5×10^6 VC/mL in 1.8 L.
6. Check the pH calibration and perform a one-point calibration offset (if required).
7. On the day of transfection, determine viability and the viable cell density. We recommend reaching $3.0 \pm 0.5 \times 10^6$ VC/mL and $\geq 95\%$ viability before proceeding with transfection.
8. Add a volume of AAV-MAX Enhancer to the bioreactor that is equal to 10 mL/L of the initial culture volume (Table 3).

Table 3. Recommended transfection parameters.

Process parameter	Target
Viable cell density (step 1)	1.5×10^6 VC/mL
Pretransfection cell density (step 7)	3.0×10^6 VC/mL
Plasmid DNA concentration*	1.5 mg/L
Viral-Plex Complexation Buffer	100 mL/L
AAV-MAX Transfection Reagent	6 mL/L
AAV-MAX Transfection Booster	3 mL/L
AAV-MAX Enhancer	10 mL/L

* Assuming a stock plasmid DNA concentration of 1 mg/mL.

9. Prepare the transfer plasmid DNA, Rep/Cap plasmid DNA, and helper plasmid DNA in your optimized ratio. Use 1.5 mg of total plasmid DNA per L of culture volume to be transfected (Table 3).
Note: This ratio can be optimized based on plasmid size and serotype.
10. Prepare the AAV-MAX Transfection Reagent and plasmid DNA complexes as indicated in Table 3.
 - a. To a 250 mL PETG Nalgene bottle, add Viral-Plex Complexation Buffer in a volume equal to 10% of the volume of culture to be transfected.
 - b. Add the total plasmid DNA from Step 9 to the Viral-Plex Complexation Buffer. Mix by gently swirling the bottle.

- c. Gently swirl the bottle containing AAV-MAX Transfection Reagent to mix.
 - d. Add a volume of AAV-MAX Transfection Booster to a sterile 50 mL polypropylene tube to obtain a final concentration of 3 mL/L in the culture to be transfected. Then add AAV-MAX Transfection Reagent to obtain 6 mL/L in the culture to be transfected. Swirl the tube gently to mix the contents and incubate at room temperature for 10 minutes.
Note: The AAV-MAX Transfection Booster and AAV-MAX Transfection Reagent complex is stable for 1 hour.
 - e. Add the mixture containing AAV-MAX Transfection Booster and AAV-MAX Transfection Reagent from Step 10d to the diluted plasmid DNA from Step 10b. Swirl gently to mix, then incubate stationary at room temperature for 10 minutes. The 10-minute room-temperature incubation, after gently swirling, is stationary; no further agitation is performed. During incubation, attach a transfer cap to the bottle and sterile-weld the tube on the bottle to the bioreactor. After incubation, use a peristaltic pump to transfer the solution to the bioreactor at a rate of ~ 70 mL/min.
Note: For optimal AAV production, we recommend 10 minutes for complexation of the transfection reagent and plasmid DNA. The entire complexation mixture should be added to the bioreactor within 15 minutes of completing the 10-minute complexation step.
11. Aseptically remove samples for analysis to determine the optimal titer-based harvest.
 - a. Measure cell density, viability, and diameter using a cell viability analyzer.
 - b. Measure pH, dissolved O_2 , and dissolved CO_2 on a gas analyzer.
 - c. Measure the metabolites on a bioanalyzer.
Note: Ensure all equipment is calibrated prior to use. Using equipment that is out of specification can negatively impact cell health and production titers.

Recommendations for scaling to larger vessels

- It is important to consider the power input per volume (P/V), tip speed, mixing time, and addition of the transfection complex. The P/V in the protocol is 20 W/m^3 , and the tip speed is 0.66 m/s. This provides a robust system at a 2 L working volume and minimizes shear from the impeller while providing optimal mixing. We recommend scaling up based on P/V if possible.
- Volumes can be scaled as a percentage of the culture volume to be transfected based on the percentages given in Table 2.
- Pay attention to the hold time and mixing of transfection reagents, DNA, and the transfection complex. Ensure proper mixing at each step and that incubation and the duration of addition is not prolonged unnecessarily.

Ordering information

Product	Cat. No.
3 L HyPerforma Glass Bioreactor, 120 V, heat only	F100-2680-002
HyPerforma G3Lab Controller for HyPerforma Glass Bioreactor, non-GMP	F100-2695-002
Gibco Viral Production Cells 2.0	A49784
Gibco Viral Production Medium	A4817902
Gibco AAV-MAX Transfection Kit	
– Gibco AAV-MAX Transfection Reagent	A50516
– Gibco AAV-MAX Transfection Booster	
– Gibco AAV-MAX Enhancer	
Viral-Plex Complexation Buffer	A4983901
GlutaMAX Supplement	35050079

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