PROTOCOL

FectoVIR®-AAV
DNA transfection reagent for virus production

DESCRIPTION

FectoVIR®-AAV is an innovative cationic nanotechnology developed for the gene and cell therapy field to improve recombinant adeno-associated virus (rAAV) production. This ready-to-use chemically defined reagent combines the flexibility of transient transfection in suspension cells with the scalability and high-speed production. It aims to enhance large scale bioproduction of rAAV through an optimized structure and improved transfection parameters.

Recommendations for optimal recombinant AAV production

Polyplus' suggestions, listed below, lead to high plasmid DNA transfection efficiency using the FectoVIR®-AAV transfection reagent and promote high rAAV titer production in suspension HEK-293 cultures.

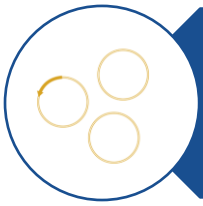
Cell density at transfection

FectoVIR®-AAV was optimized using a cell density of 2 x 10^6 cells/mL at the time of transfection. Passage cells to reach the recommended cell density with cells in exponential growth phase.

If a high cell density system is used, some of the parameters must be adjusted accordingly (Refer to page 10).

DNA purity

Use high-quality plasmid preparation, free of proteins and RNA. Plasmid DNA solutions characterized by an $OD_{260/280}$ > 1.8 are suitable.



rAAV coding plasmid

FectoVIR®-AAV was optimized for rAAV production using a 2:2:1 plasmid ratio of transfer plasmid (pAAV-GFP), packaging plasmid and helper plasmid.

Optimize the plasmid ratio according to your process (Refer to page 10).

DNA amount

FectoVIR®-AAV was optimized for rAAV production using 1 µg of DNA per 10^6 cells.

DNA to FectoVIR®-AAV ratio

FectoVIR®-AAV was optimized for rAAV production using a DNA : FectoVIR®-AAV ratio of 1 µg : 1 µL.

Complexation volume

FectoVIR®-AAV was optimized for rAAV production using a 5% complexation volume.

1. Transfection protocol

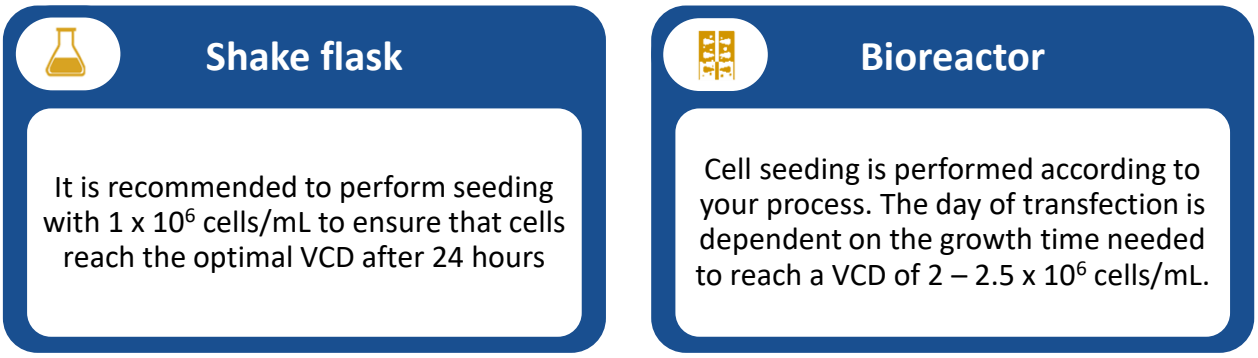
FectoVIR®-AAV is perfectly suited for DNA transfection of cells grown in suspension in shaker flasks, spinners, cell culture bags or bioreactors in serum-free media. FectoVIR®-AAV is compatible with the use of antibiotics in the cell culture medium.

1.1. Cell seeding

On the day of cell seeding, adjust cell density according to your process to reach the exponential growth phase with a viable cell density (VCD) around 2 – 2.5 x 10^6 cells/mL at time of transfection.

Cell seeding must be adjusted according to your cell culture volume (Figure 1).

Figure 1. Recommendations for cell seeding according to the cell culture volume.



***On the day of transfection**, VCD must be determined to adjust the DNA amount used for transfection.*

1.2. Preparation of the complexes and transfection

The following protocol is given for the transfection of plasmids coding for rAAV into suspension cells. For co-transfection of multiple plasmids, the suitable plasmid ratio depends on the size of the plasmids, the plasmid constructs, and the desired expression level of each plasmid. Please adjust the ratios according to your application.

The different complexation parameters are described in Table 1. For each parameter, we recommend a specific condition that may be further optimized according to your process.

Table 1. Complexation parameters.

Parameter	Recommended condition	Range of optimization
DNA amount (per 10^6 cells)	1 µg DNA	0.5 µg – 2 µg
Ratio (µg DNA : µL FectoVIR®-AAV)	1:1	1:0.75 – 1:2
Complexation volume (% total culture volume)	5%	1% - 10%
Complexation time	30 min	15 min – 60 min
Complexation medium* (without supplements)	DMEM high glucose	Own culture medium, DMEM low glucose, Freestyle™ F17, BalanCD™ HEK-293, PBS

Allow all the components to equilibrate to room temperature before starting the transfection protocol.

** The complexation medium should contain neither Pluronic® F-68/Poloxamer 188/Kolliphor® P188 nor antibiotics.*

If a higher cell density is used, it might be difficult to decrease the complexation volume up to 1%.

1.3. Transfection protocol

Transfection parameters (*i.e.*, DNA amount and FectoVIR®-AAV volume) should be adjusted according to the cell density reached at the time of transfection. Based on the recommended/standard conditions (Table 1), please find in the Table 2 few examples of DNA amounts and FectoVIR®-AAV volumes according to the cell density at the time of transfection.

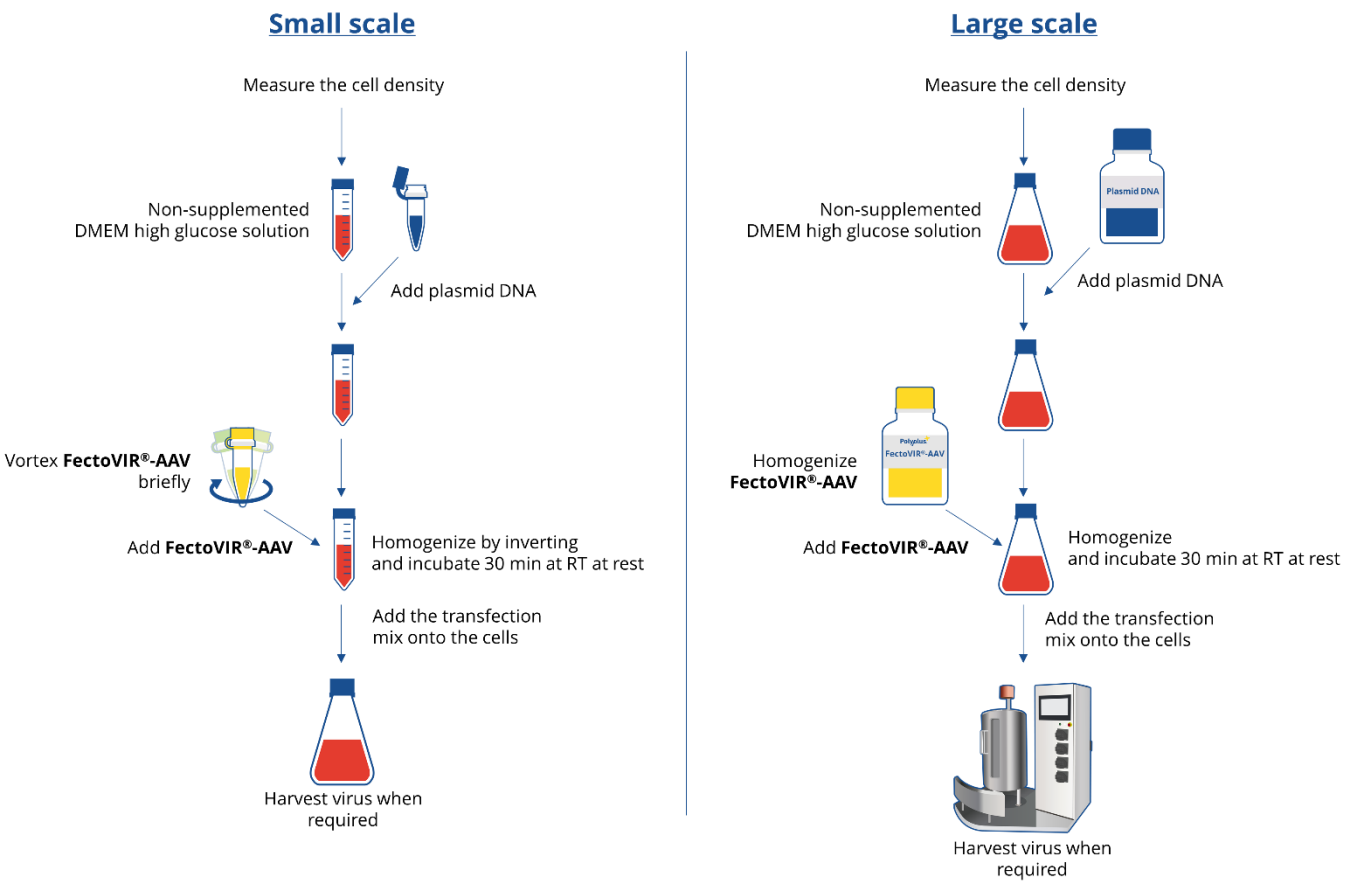
Table 2. DNA transfection guidelines according to the culture parameters used.

Cell culture parameters			Transfection parameters		
[Cell density]	Cell culture volume	Total cell density	Volume of complexation medium	DNA amount	Volume of FectoVIR®-AAV
2 x 10^6 cells/mL	30 mL	60 x 10^6 cells	1.5 mL	60 µg	60 µL
	1 L	2 000 x 10^6 cells	50 mL	2 mg	2 mL
	100 L	200 000 x 10^6 cells	5 L	200 mg	200 mL
2.5 x 10^6 cells/mL	30 mL	75 x 10^6 cells	1.5 mL	75 µg	75 µL
	1 L	2 500 x 10^6 cells	50 mL	2.5 mg	2.5 mL
	100 L	250 000 x 10^6 cells	5 L	250 mg	250 mL

The following protocol is given for transfection in 30 mL (small scale) of cell culture medium according to the recommended/standard conditions.

1. On the day of transfection, measure the cell density and determine transfection parameters (DNA amount and FectoVIR®-AAV volume per million cells) according to Table 1 & 2.
2. Prepare 1.5 mL of non-supplemented DMEM high glucose corresponding to 5% of the total volume of culture.
3. Dilute each rAAV coding plasmid in the non-supplemented DMEM high glucose solution.
4. Vortex FectoVIR®-AAV briefly.
5. Add the corresponding volume of pure FectoVIR®-AAV onto the diluted DNA solution all at once.
6. Homogenize immediately the complex solution by inverting the vessel 3-4 times.
7. Incubate for 30 minutes at room temperature and at rest.
8. Add the transfection mix onto the cells.
9. Incubate cells at appropriate temperature, shaking and CO2 levels (*e.g.*, 37°C, 130 rpm, 8%) and harvest virus when required (*e.g.*, 72 h post-transfection).

Optimize the harvest time according to your process.



If the protocol is used at large scale, the time of homogenization should not exceed 1 minute, and the complexation should be performed at rest.

Some serum-free media are not permissive to transfection. Please ensure that the medium you are using is permissive to transfection and suitable for high transfection efficiency. Feel free to contact Polyplus scientific support online for tips and advice: support@polyplus-transfection.com.

1.4. Design of experiment (DOE)

To determine the optimum transfection conditions for rAAV production according to your process, Polyplus recommends using a 2-factors central composite design (CCD) and a response surface methodology (RSM). RSM is a useful method to study the effect of several transfection parameters influencing the rAAV production by varying them simultaneously and carrying out a limited number of experiments. The DNA amount and the DNA : FectoVIR®-AAV ratio are the two main factors affecting the yield of transfection. Accordingly, we suggest using a CCD comprising a total of 13 experiments: a full factorial design with 2^2=4 points (coded at ± a), 4 axial points (coded as $\alpha = \pm 1$) representing extreme values for estimation of the model curvature and 5 central points (coded at 0) repeated to consider the experimental error, as summarized in Figure 2 and Table 3. The collected data are processed by statistical software.

Figure 1. Central composite design with two factors.

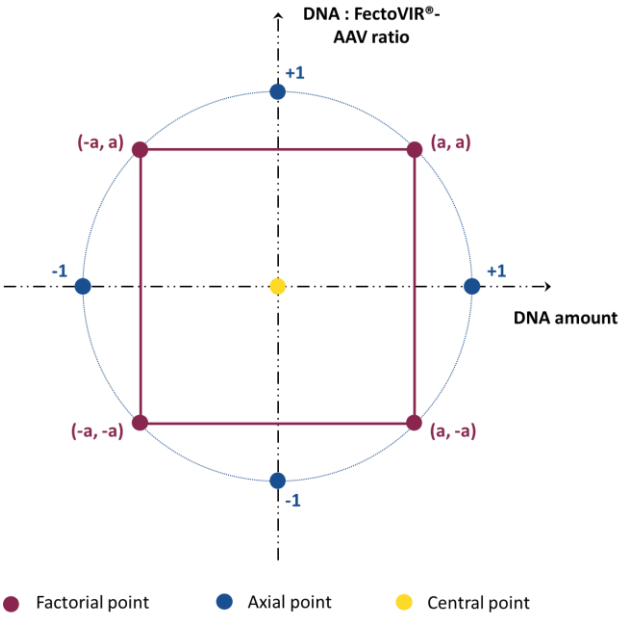


Table 3. Parameters of experimental design.

Parameter	Value
Design type	Central composite
Number of factors	2
Number of responses	1
Axial distance α	1
Replicates of factorial points	1
Replicates of axial points	1
Replicates of center points	5
Total number of experiments	13

For both of the studied factors (DNA amount and DNA : FectoVIR®-AAV ratio), the minimum and maximum levels are determined as the extreme values of the range of optimization and the central point is selected as the recommended protocol condition (Table 1), as illustrated in the Table 4.

Table 4. Levels of factors used in the recommended experimental design.

Factors	Levels				
Coded	Axial (-1)	Low (-a)	Central (0)	High (+a)	Axial (+1)
DNA amount (µg per 10^6 cells)	0,2	0,43	1	1,57	1,8
DNA : FectoVIR®-AAV ratio (µg : µL)	0,5	0,65	1	1,35	1,5

The selected factor combinations indicating the actual and coded levels of the experimental design are randomized and represented in Table 4.

Extreme values for both transfection parameters are determined to ensure equidistant points from the central one.

Table 5. List of experiments planned in the recommended CCD designed to optimize rAAV production.

Number	Coded levels		Uncoded levels	
	DNA amount	DNA : FectoVIR®-AAV ratio	DNA amount	DNA : FectoVIR®-AAV ratio
1	0	0	1,00	1,00
2	a	-a	1,57	0,65
3	-a	a	0,43	1,35
4	0	-1	1,00	0,50
5	1	0	1,80	1,00
6	0	0	1,00	1,00
7	-1	0	0,20	1,00
8	0	0	1,00	1,00
9	0	1	1,00	1,50
10	0	0	1,00	1,00
11	0	0	1,00	1,00
12	-a	-a	0,43	0,65
13	a	a	1,57	1,35

For beginners, it corresponds to a standard DOE to optimize two main factors (i.e. DNA amount and FectoVIR®-AAV volume). Once these conditions are determined, it is possible determine the optimal transfection conditions by designing another experience that involves two other main factors (i.e. complexation volume and incubation time). However, it is first suitable to determine all interactions involved in the transfection process with these four factors by using a factorial design. It is then possible to optimize each parameter with a CCD according to the interaction between factors.

1.5. Recombinant AAV production analysis

There are several methods of titration that can be used to determine the viral titer of a sample. It is recommended to measure both physical and functional titer for accurate viral titration.

The determination of the physical titer that measure the concentration of viral particles in a sample is based on the presence of a viral capsid protein or a viral nucleic acid.

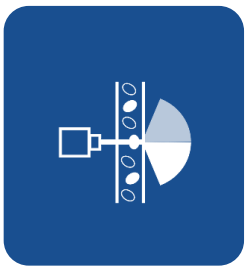
The determination of the functional titer, which measures how many produced viral particles can actually infect cells, involves the infection of a target cell line with the recombinant virus followed by an expression assay of a gene carried on the transfer plasmid.

qPCR - Viral genomes

Once AAV are harvested, the genomic DNA is extracted using a viral DNA extraction kit. The number of viral copies is quantified by the SYBR-Green qPCR method. Primers targeting the gene of interest (GOI) or "universal" primers can be used (Aurnhammer et al., 2012).

ELISA - Viral capsids

Once AAV are harvested, the number of viral capsids is quantified by a sandwich ELISA technique. The titration of AAV capsids by ELISA can be performed using kits, following the manufacturer's instructions.



Infection/Transduction & Flow cytometry - Infectious particles

Once AAV are harvested, a permissive cell line is transduced with the rAAV produced. Adherent HEK-293 cells are infected with serial dilutions of rAAV vectors. 72h post-transduction, cells are harvested and flow cytometry can be used to count the number of target cells that are positive for vector encoded transgene GFP expression. The number of fluorescent cells correspond to the number of transduced cells.

Feel free to contact Polyplus scientific support online for tips and advice: support@polyplus-transfection.com.

2. Troubleshooting

Observations	Actions
Low viral titer	• Optimize the FectoVIR®-AAV to DNA ratio starting from 0.5 µL FectoVIR®-AAV/µg DNA up to 2 µL FectoVIR®-AAV/µg DNA. • Optimize the amount of plasmid DNA starting from 0.2 µg to 1.8µg. • Optimize the ratio between the different plasmids used. • Ensure that the serum-free medium is permissive to transfection. • Use a positive control such as a plasmid encoding for a common reporter gene (Luciferase, GFP, etc.). • Use high-quality plasmid preparation, free of proteins and RNA ($OD_{260/280}$ > 1.8). • Precipitate formation may appear during complexation when excess DNA (high cell density at transfection) and low complexation volume is used. Increase the volume of complexation or decrease DNA amount to avoid precipitation. ! *Turbidity is different from precipitation and does not affect transfection.*
Cellular toxicity	• Optimize the DNA: FectoVIR®-AAV ratio by decreasing the volume of FectoVIR®-AAV. • Check the DNA concentration and decrease the amount of plasmid DNA used, keeping the DNA: FectoVIR®-AAV ratio constant. • On the day of cell seeding, prepare the cell suspension by centrifuging the cells and resuspending them in fresh, pre-warmed serum-free medium. • Make sure that the plasmid preparation is endotoxin-free.
Scale-up concerns	• Contact us online for tips and advice: support@polyplus-transfection.com

3. Product information

3.1. Ordering information

Part N°	FectoVIR®-AAV Transfection Reagent
101000044	1 mL
101000022	10 mL
101000004	100 mL
101000054	1 L

3.2. Content

100 mL of FectoVIR®-AAV transfection reagent is sufficient to transfect on average 50 L of cell culture.

3.3. Reagent use and limitations

For bioproduction and research use only. Not intended for animal or human diagnostic or therapeutic use.

3.4. Quality control

All lots of FectoVIR®-AAV are tested during and after manufacturing to guarantee accurate chemical composition and to ensure constant quality and lot-to-lot reproducibility. FectoVIR®-AAV potency is evaluated in a DNA transfection experiment of HEK-293 cells.

The provided FectoVIR®-AAV Certificate of Analysis displays detailed results of the lot release Quality Controls (QCs).

3.5. Formulation and storage

FectoVIR®-AAV is chemically defined and guaranteed free of animal origin products.

FectoVIR®-AAV should be stored at 5 ± 3 °C to ensure long term stability. FectoVIR-AAV®, as guaranteed and indicated on the Certificate of Analysis, is stable for 24 months from the manufacturing date when stored appropriately.

Polyplus-transfection® has been awarded ISO 9001 Quality Management System Certification since 2002, which ensures that the company has established reliable and effective processes for manufacturing, quality control, distribution, and customer support.

3.6. Trademarks

Polyplus-transfection and FectoVIR-AAV are registered trademarks of Polyplus-transfection.

Pluronic is a registered trademark of BASF. FreeStyle is a trademark of Life Technologies Corporation. BalanCD is a trademark of Irvine Scientific Sales Company, Inc.

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3.7. Contact information

Do you have any technical question regarding your product?

- Website: www.polyplus-transfection.com
- Email: support@polyplus-transfection.com
- Phone: +33 3 90 40 61 87

Contact the friendly Scientific Support team which is composed of highly educated scientists, PhDs and Engineers, with extensive hands-on experience in cell culture and transfection. The Scientific Support is dedicated to help our customers reach their goals by proposing different services such as: protocol optimization, personalized transfection conditions, tailored protocols, etc.

For any administrative question, feel free to contact our administration sales team:

- Reception Phone: +33 3 90 40 61 80
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Please note that the Polyplus-transfection® support is available by phone from 9:00 am to 5:00 pm CEST.