PROTOCOL

FectoVIR®-LV
DNA transfection reagent for virus production

DESCRIPTION

FectoVIR®-LV is a novel class of animal free transfection reagent specifically developed for industrial scale production of lentiviral vectors (LVV) in suspension HEK-293 cell types. FectoVIR®-LV transfection reagent guarantees higher LVV viral titers and scalability for industrial scale manufacturing resulting in a process cost economics.

1. Transfection protocol for suspension cell system

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

Polyplus highly recommends performing a DOE study to obtain excellent conditions in a short time. Please refer to section 1.5 for more details or contact support@polyplus-transfection.com.

1.1. Recommendation for optimal recombinant LV production

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

Cell density at transfection

FectoVIR®-LV 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.

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

rLV coding plasmid

FectoVIR®-LV was optimized for rLV production using a 1:2:1:3 (pREV, pGag:Pol, pENV, pGOI)

Optimize the plasmid ratio according to your process (Refer to Troubleshooting section).

DNA amount

FectoVIR®-LV was optimized for rLV production using 1 µg of DNA per 10^6 cells

DNA to FectoVIR®-LV ratio

FectoVIR®-LV was optimized for rLV production using a DNA : FectoVIR®-LV ratio of 1 µg : 1 µL

Complexation volume

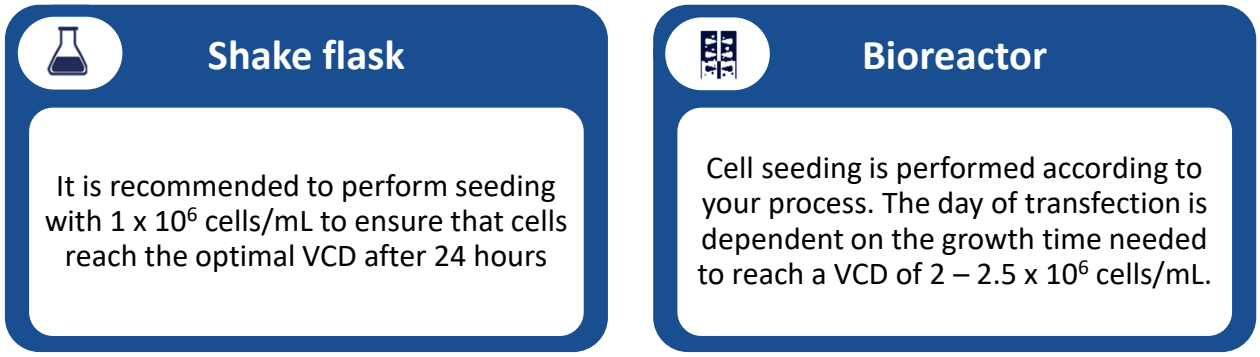
FectoVIR®-LV was optimized for rLV production using a 5% complexation volume

1.2. 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.*
VCD at transfection may be optimized (could be different 2 M cells/mL)

Of note, FectoVIR®-LV was shown to work quite well in perfusion systems, with up to 10 x 10^6 cells/mL at the time of transfection. If you are interested in evaluating this option, feel free to contact us at support@polyplus-transfection.com

1.3. Preparation of the complexes and transfection

The following protocol is given for the transfection of plasmids coding for rLV 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. Recommended starting conditions and ranges of optimization for transfection parameters.

Parameter	Recommended condition	Range of optimization
VCD at the time of transfection	2 – 2.5 x 10^6 cells/mL	1 – 4 in batch production
DNA amount (per 10^6 cells)	1 µg DNA	0.5 µg – 2 µg
Ratio (µg DNA : µL FectoVIR®-LV)	1:1	1:0.5 – 1:2
Complexation volume (% total culture volume)	5%	2.5% - 10%
Complexation time	15 min	5 min – 45 min
Complexation medium* (without supplements)	Freestyle™ F17	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.*
* FectoVIR®-LV *is compatible with expression enhancers (eg. Sodium butyrate, Valproic Acid).*

FectoVIR®-LV is compatible with perfusion system using higher cell density, please contact support@polyplus-transfection.com for more information.

1.4. Transfection protocol

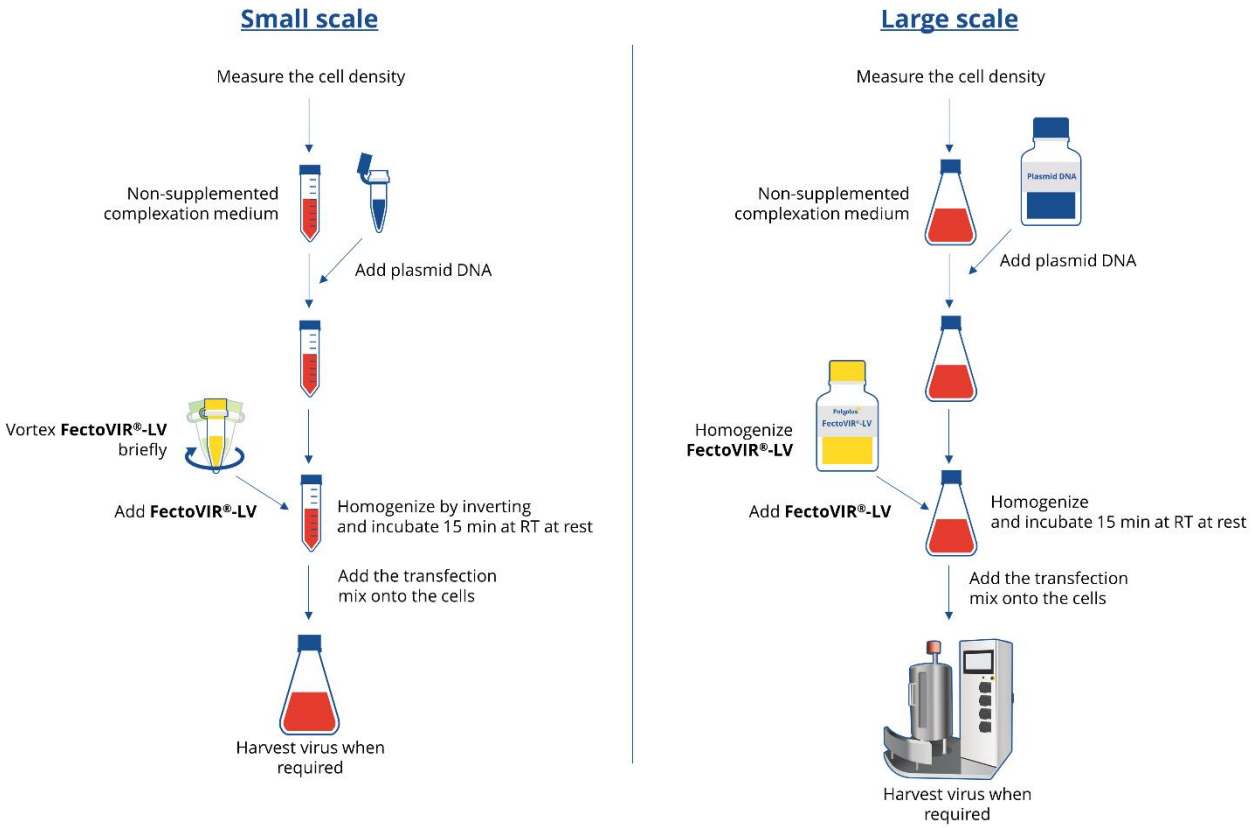
Transfection parameters (*i.e.*, DNA amount and FectoVIR®-LV volume) should be adjusted according to the cell density reached at the time of transfection. Based on the recommended/standard conditions (Table 1), please find in the Table 2 few examples of DNA amounts and FectoVIR®-LV volumes according 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®-LV**
2×10^6 cells/mL	30 mL	60×10^6 cells	1.5 mL	60 µg	60 µL
	1 L	$2\,000 \times 10^6$ cells	50 mL	2 mg	2 mL
	100 L	$200\,000 \times 10^6$ cells	5 L	200 mg	200 mL
2.5×10^6 cells/mL	30 mL	75×10^6 cells	1.5 mL	75 µg	75 µL
	1 L	$2\,500 \times 10^6$ cells	50 mL	2.5 mg	2.5 mL
	100 L	$250\,000 \times 10^6$ 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®-LV 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 rLV coding plasmid in the non-supplemented DMEM high glucose solution.
4. Vortex FectoVIR®-LV briefly.
5. Add the corresponding volume of pure FectoVIR®-LV onto the diluted DNA solution all at once.
6. Homogenize immediately the complex solution by inverting the vessel 3-4 times.
7. Incubate for 15 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.

1.5. Experimental design proposal

To gain precious time, we recommend using Design of Experiment (DoE) for your evaluation.

During transfection, different factors will impact the complexation leading to impact transfection efficiency and titers. Polyplus has identified several factors such as DNA amount and DNA:Reagent ratio which are the most critical factors, but few others may also affect the process such as the VCD at transfection, complexation time and complexation volume. Plasmid ratio is also a key parameter that should be explored during optimization of the process.

Table 3 : Key parameters for transfection to consider using a DoE approach

Parameters	Minimum	Maximum
VCD at the time of transfection (10^6 cells per mL)	2	4 (batch process) 10 (perfusion system)
DNA amount (µg per 10^6 cells)	0.5	2.0
Ratio (µg DNA : µL FectoVIR®-LV)	1:0.5	1:2
Complexation volume (% total culture volume)	2.5	10
Complexation time (min)	5	45
Complexation medium* (without supplements)	Freestyle™ F17, Own culture medium, DMEM low glucose, BalanCD™ HEK-293, PBS	

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

We propose the following experimental plan using shake flask (125 mL) or mini-bioreactors to screen the optimal condition for transfection:

Table 4 : Experimental plan proposal

Exp No	Priority	Run Order	Cell density (E6 cells/mL)	DNA amount (µg/mL)	FectoVIR®-LV volume (µL/mL)	DNA/million cells	DNA : FectoVIR®-LV ratio
1	1	2	2.5	2	2	0.8	1 : 1
2	1	3	2.5	2	6	0.8	1 : 3
3	1	6	2.5	4	2	1.6	1 : 0.5
4	1	8	2.5	4	6	1.6	1 : 1.5
5	1	15	2.5	3	4	1.2	1 : 1.33
6	1	7	2.5	2	2	0.8	1 : 1
7	1	10	2.5	2	6	0.8	1 : 3
8	1	13	2.5	4	2	1.6	1 : 0.5
9	1	5	2.5	4	6	1.6	1 : 1.5
10	1	11	2.5	3	4	1.2	1 : 1.33
11	1	14	2.5	2	2	0.8	1 : 1
12	1	12	2.5	2	6	0.8	1 : 3
13	1	4	2.5	4	2	1.6	1 : 0.5
14	1	9	2.5	4	6	1.6	1 : 1.5
15	1	1	2.5	3	4	1.2	1 : 1.33
16	2	19	2.5	3	1.17	1.2	1 : 0.39
17	2	24	2.5	3	6.83	1.2	1 : 2.28
18	2	28	2.5	1.59	4	0.64	1 : 2.52
19	2	16	2.5	4.41	4	1.76	1 : 0.91
20	2	29	2.5	3	4	1.2	1 : 1.33
21	2	20	2.5	3	1.17	1.2	1 : 0.39
22	2	23	2.5	3	6.83	1.2	1 : 2.28
23	2	17	2.5	1.59	4	0.64	1 : 2.52
24	2	26	2.5	4.41	4	1.76	1 : 0.91
25	2	18	2.5	3	4	1.2	1 : 1.33
26	2	30	2.5	3	1.17	1.2	1 : 0.39
27	2	22	2.5	3	6.83	1.2	1 : 2.28
28	2	21	2.5	1.59	4	0.64	1 : 2.52
29	2	27	2.5	4.41	4	1.76	1 : 0.91
30	2	25	2.5	3	4	1.2	1 : 1.33

Important notes regarding this design:

This two-factors central composite design corresponds to 30 independent flasks/mini-bioreactors, with three replicates for each condition. All other controlled parameters in the experiment should be set and maintained constant across the experiment (incubation time, cell density, complexation volume and medium, etc…).

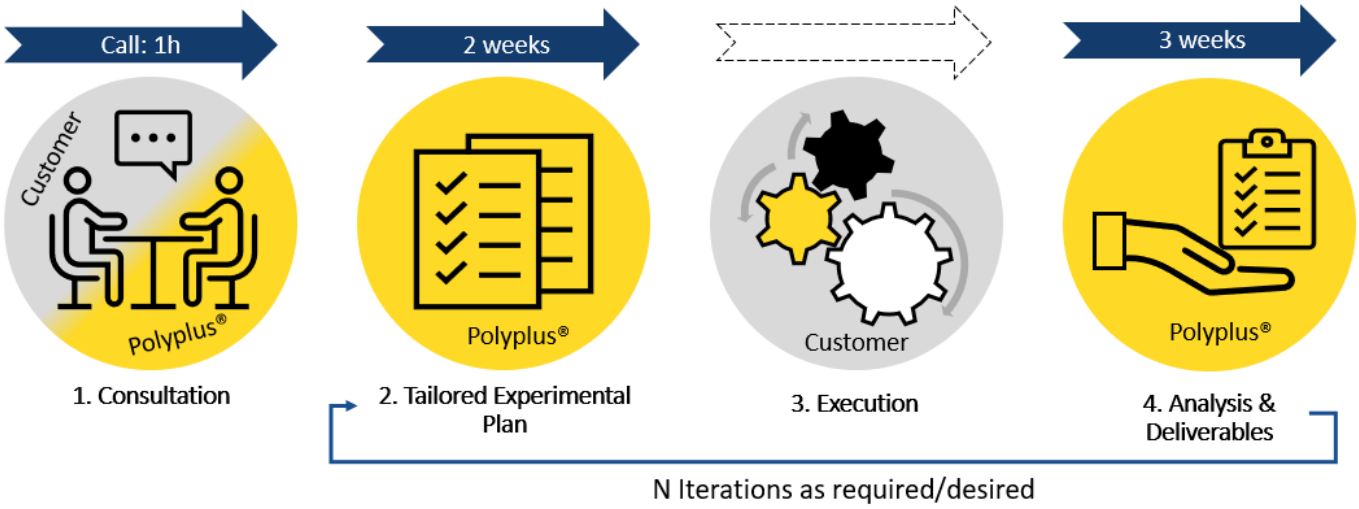
If 30 flasks are too much for one operator to handle in one experiment, it is possible to separate this design in two parts. We recommend running Experiments 1-15 first (Priority 1) as these could be analyzed separately to give a first idea of the capacities of your system. For better prediction power over the design space, we recommend however to run experiments 16-30 (Priority 2) in a second run to complement the design.

Please note that one of the main key concept DoE relies on is randomization. Therefore, it is important to run these different experiments following the randomized order assigned in the above table. If you run the 30 flasks in just one day, we recommend using your own randomized order that does not take into account the Priority 1/Priority 2 separation.

Feel free to contact Polyplus scientific support if you have any questions prior to performing this experiment: support@polyplus-transfection.com.

1.6. Design of experiment (DOE) service

Polyplus now proposes a service of Design of Experiment allowing FectoVIR®-LV customers to optimize titers and transfection/bioprocess parameters in a record time.
The service is managed by our scientific support experts and consists in a sequential stage process described in the following scheme:



To access this service, feel free to contact support@polyplus-transfection.com.

2. Recombinant LV production analysis

The two most popular assays to quantify LVV productivity are:

- Infectivity test to determine functional titers

The determination of the functional titer, which measures how many produced viral particles can 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.

- ELISA (p24) to determine the number of viral particules

Commercial kit is available on the market.

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

3. Troubleshooting

Table 13: Troubleshooting for Suspension and Adherent cell systems

Observations	Actions
Low viral titer	• Ensure that the complexation 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.* • Optimize the FectoVIR®-LV to DNA ratio starting from 0.5 µL FectoVIR®-LV /µg DNA up to 2 µL FectoVIR®-LV /µg DNA. • Optimize the amount of plasmid DNA starting from 0.5 µg to 2 µg. • Optimize the ratio between the different plasmids used. *Of note, we recommend using DoE to optimize transfection with FectoVIR®-LV. Feel free to contact support@polyplus-transfection.com for DoE help.*
Cellular toxicity	• Optimize the DNA:FectoVIR®-LV ratio by decreasing the volume of FectoVIR®-LV • Check the DNA concentration and decrease the amount of plasmid DNA used, keeping the DNA:FectoVIR®-LV 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

4. Product information

4.1. Ordering information

Part N°	FectoVIR®-LV Transfection Reagent
101000187	1 mL
101000188	10 mL
101000189	100 mL

4.2. Reagent use and limitations

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

4.3. Quality control

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

The provided FectoVIR®-LV Certificate of Analysis displays results of the lot release Quality Controls.

4.4. Storage & Expiry

FectoVIR®-LV should be stored at 5 ± 3 °C to ensure long term stability. Expiry date is indicated in the certificate of analysis.

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.

4.5. 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
- Fax: +33 3 90 40 61 81
- Addresses:

Polyplus® locations	Address
Headquarter Transfection reagent manufacturing site	75, rue Marguerite Perey 67400 Illkirch France
Plasmid design site	80 Rue du Dr Yersin 59120 Loos France
US sales office	1251 Ave of the Americas 34th fl. New-York - NY 10020 United States
Chinese sales office	Room 1506, Tower B, Sunyoung Center No. 28 Xuanhua Road Changning District, Shanghai China

Please note that the Polyplus® support is available by phone from 9:00 am to 5:00 pm CEST.