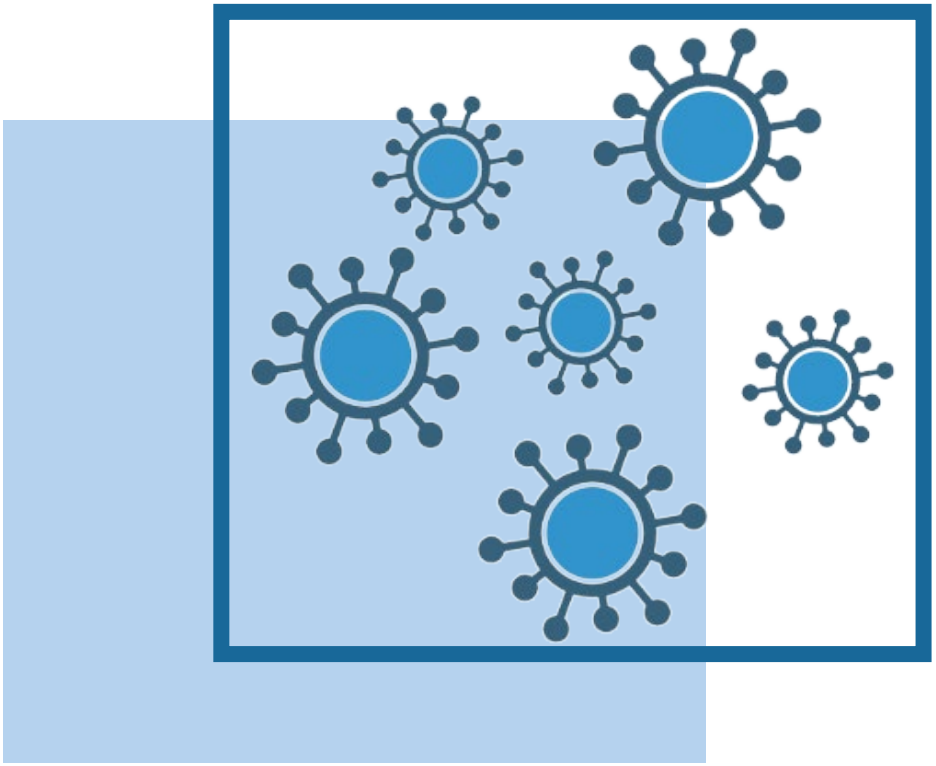
HYVIR™ TRANSFECTION REAGENT

INSTRUCTION MANUAL



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For Lentiviral Production

Reach high-titer virus production

Instruction Manual

Description

HYVIR™ transfection kit for lentiviral production in HEK-293T

Catalog Number	Description	Volume (µL)	HYVIR Boost 100X Volume (mL)	Number of Transfection in 24-well plate[1]	Number of Transfection in 100mm plate[1]
HYV01500	HYVIR™	1500	5	1600	160
HYV03000	HYVIR™	2 x 1500	2 x 5	3200	330
HYV15000	HYVIR™	15 000	50	/	/
HYV30000	HYVIR™	2 x 15 000	2 x 50	/	/

[1] Values are given for transfections using the recommended transfection conditions (0.3 µg DNA/well and 3 µg DNA/100mm dishes)

1. Technology

1.1. Description

HYVIR™, is a novel lipid-based transfection kit dedicated to enhance viral particles production in HEK-293T. Its specific formulation allows an optimal plasmid compaction, protection and delivery rendering HYVIR ideal for the production of last generation of lentivirus. 3rd generation system while improving the safety of the 2nd generation by splitting the packaging system into two plasmids also increases the difficulty for delivery: now 4 plasmids need to be transfected at the same time under a precise stoichiometry. Thanks to its chemical moieties, HYVIR is the perfect transfection reagent dedicated to this task as it allows maximal compaction for an efficient co-transfection of packaging plasmid(s), envelope plasmid and lentiviral transfer plasmid encoding your gene of interest.

Issued from the catalogue of proprietary last generation of lipids from OZ Biosciences, this reliable and reproducible transfection reagent is easily scalable for production in large volumes and can be raised to superior grade.

1.2. Storage and shipping condition

Storage: Upon reception and for long-term use, store HYVIR at 4°C and HYVIR Boost (100X) at -20°C.
Stability: 1 year
Shipping condition: The reagent is shipped at RT

2. Applications and Protocols

2.1. General Considerations

- Allow reagents to reach room temperature before starting
- It is mandatory to use medium without any supplement for the preparation of complexes. Culture mediums such as DMEM (with or w/o phenol red), RPMI (with or w/o phenol red), DMEM-F12, alpha-MEM, EMEM or OptiMEM are recommended
- A medium change post-transfection is not recommended since it could dramatically affect viral production.

- Collect supernatant containing lentiviral particles 48H to 72H after transfection. Ideally preform two collects at 48H and 72H and use the Viro-PEG lentivirus concentrator kit (ref #LVG500) to increase viral titer. Refer to Concentration paragraph for more info.

2.2. Important Guidelines

- **Cells** should be healthy and assayed during their exponential growing phase. The presence of contaminants (mycoplasma, fungi) will considerably affect the transfection efficiency. Use regularly passaged cell lines at a density of 80-90% confluence (visual confluence) at the time of transfection; low density could result in toxicity and low virus titer.

- **Nucleic Acids** should be as pure as possible and free of contaminants. We suggest avoiding long storage of the diluted nucleic acids solution before the addition of HYVIR to circumvent any degradation or surface adsorption. Any generation of lentiviral system is compatible with HYVIR.

- **Culture Medium**. The exclusion of antibiotics from the media during transfection has been reported to enhance gene expression levels. We did not observe a significant effect of the presence or absence of antibiotics with the HYVIR transfection reagent.

- **Transfection conditions** depending on the final titer, it is possible to vary volume of transfection reagent or total DNA amount to increase production – refer to optimization paragraph. Be sure to use the ideal ratio for each plasmid composing the lentiviral expression system for an optimal viral production.

2.3. Cell Preparation

Cell culture prior to transfection: one day before transfection prepare the cells according to the Table 1.

It is recommended to plate the cells the day before in classical complete culture medium so that they are 80-90 % confluent at the time of transfection to ensure a maximum of viability and viral production. Adjust cell number and culture volume accordingly to the surface area of the culture dish.

Table 1: Cell number suggested (per well).

Tissue Culture Dish format	Surface area per well[1]	Cell Number	Culture medium (mL)	Vol. for complex (µL)
24 wells	2 cm^2	5 x 1.10^4	0.4	100
10-cm dish	55 cm^2	2 x 1.10^6	10	200

[1] Surfaces area may vary depending on the manufacturer.

Perform a medium change 1H before transfection.

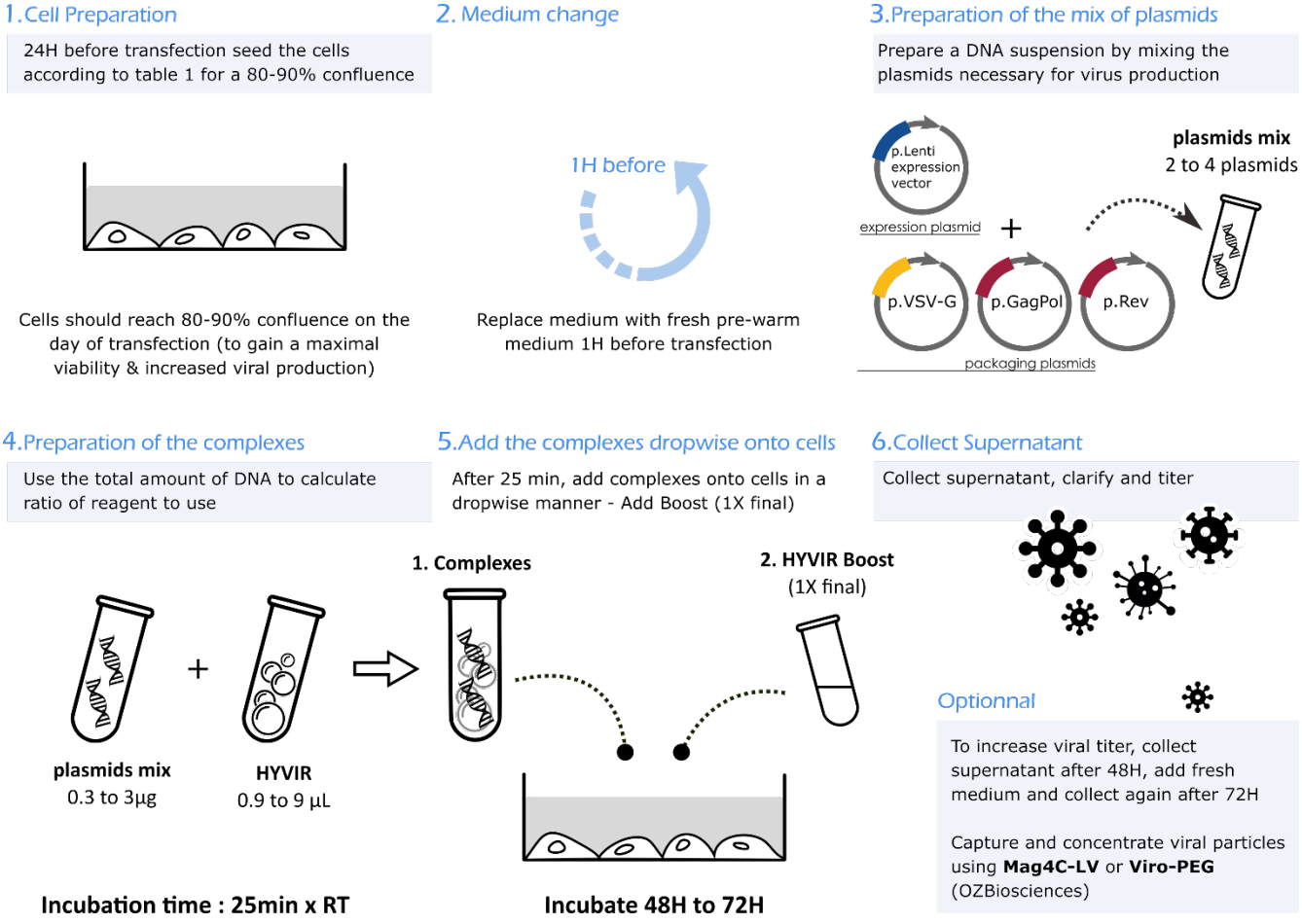
Note: expression of the vesicular stomatitis virus (VSV) may induce syncytium formation and giant cell formation (cell-cell fusion) that does not impair high viral yields (Roberts PC. *et al.* J Virol, 1999).

2.4. Standard Protocol

The following protocol sets the basis to produce lentiviral particles in 24-well plates as well as 10cm cell culture dishes using HYVIR, refer to table 2 below for recommended DNA amount and reagent volumes. Refer to paragraph 2.6 to optimize transfections.

	DNA quantity	HYVIR volume:	Total culture + transfection volume:	Boost volume (1X final):
24-well plate	0.3 µg (50 µL)	0.9 µL (50 µL)	500 µL	5 µL
10cm dish	3 µg (100 µL)	9 µL (100 µL)	10 mL	100 µL

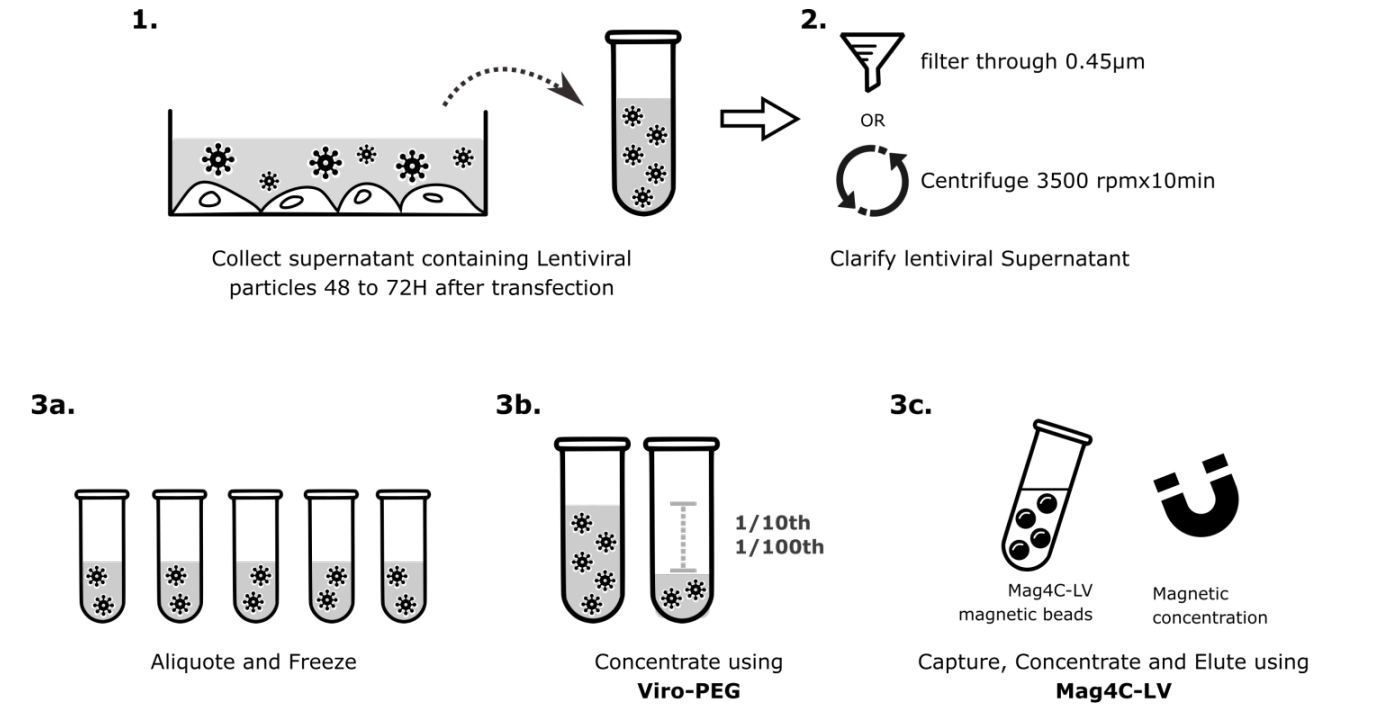
Table 2: Recommended volumes of HYVIR depending on the nucleic acid to use



1) Plate the cells **24H** before the experiment to reach **80-90%** confluence on the day of transfection
2) Replace medium with fresh pre-warm medium **1H** before transfection
3) Prepare a mix of plasmids by composing packaging and expression plasmids depending on their recommended stoichiometry
4) Add **0.3** to **3 µg** of total plasmid mix to **50** to **100 µL** medium without any supplement
5) Add **0.9** to **9 µL** HYVIR to **50** to **100 µL** medium without any supplement
6) Form complexes by combining DNA and HYVIR solutions
7) Incubate **25 min** at **RT**
8) After incubation, add complexes directly to cells in a dropwise manner and supplement with boost (**1X final**)
9) Incubate **48** to **72 H** and proceed to harvest and concentration or storage of Lentivirus

NOTE: do not perform a medium change after 24 H as this would reduce the viral titer.

2.5. Harvest, Concentrate or Store freshly produced lentivirus



Use **Mag4C-LV Conservation Buffer** for long-term storage at -80°C

1) Harvest cell supernatant **48** to**72 H** after transfection
2) Centrifuge supernatant **3500 rpm** x **10 min** or use **0.45 µm** PVDF filter to remove cell debris.
3) **3a** - Prepare aliquots of supernatant and directly freeze lentivirus in cryogenic tubes at **-80°C** or use **Mag4C conservation buffer** (LVB1000, OZ Biosciences) for long term storage.
   OR
   3b – Concentrate lentiviral particles using **Viro-PEG lentivirus concentrator** (#LVG500, OZ Biosciences):
   a. Add 1 volume of Viro-PEG to 4 volumes of clarified supernatant
   b. Mix well and refrigerate 4H to Overnight
   c. Centrifuge **3000*xg*** x **60 min** x **4°C**
   d. Remove supernatant and resuspend pellet in 1/10th to 1/100th of the original volume or in Mag4C conservation buffer.

   OR
   3c – Capture and Concentrate lentiviral particles using **Mag4C-LV kit** (#LTK11000, OZ Biosciences):
   a. Add Mag4C-LV magnetic nanoparticles to clarified supernatant
   b. Incubate at room temperature
   c. Use magnetic field to rapidly and efficiently concentrate viral particles.
   d. Remove supernatant and resuspend pellet in 1/10th to 1/100th of the original volume or in Mag4C conservation buffer or elute viral particles.
4) Optionally add **Mag4C-LV conservation buffer** 1X final (#LVB1000, OZ Biosciences) for long term storage at -80°C.

2.6. Optimization protocol

Depending on the cells, the culture conditions and on the lentiviral expression system (number of plasmids, length...), ideal conditions for transfection may vary and an optimization procedure may be needed. To achieve the highest efficiency of production, optimize the transfection conditions as follows:

- Vary the HYVIR (µL) / DNA (µg) ratio from 2:1 to 4:1.
  We recommend trying 2.0, 2.5, 3.0, 3.5 µL and 4.0 µL HYVIR per µg DNA.

- Once the optimal HYVIR/DNA ratio is found, adjust the DNA quantity according to Table 3.

- Finally, culture medium composition (for preparing the complexes), cell density, total culture medium volume and incubation times can also be optimized.

	Surface area (cm²)	**HYVIR volumes:**
24-well plates	2	0.1 to 0.5 µg
10 cm dishes	55	1 to 5 µg

Table 3: suggested range of DNA amounts for optimization

- For other culture vessel format, adjust DNA amount and HYVIR volume in proportion to the area of the well.

2.7. Increasing Viral Titer

Once the ideal conditions are found (refer to paragraph 2.6 above), it is still possible to increase final viral titer by performing 2 collects instead of one.

This procedure leads to two times diluted viral suspension; we thus recommend using the **Viro-PEG** lentivirus concentrator kit (OZ Biosciences, #LVG500) to concentrate viral particles produced.

1) 36 to 48H after transfection withdraw cell culture medium containing viral particles and add fresh pre-warm medium – Do not discard supernatant!
2) Clarify the supernatant by filtering through 0.45µm or by centrifugation
3) Store the viral particles
4) After 72H, harvest the supernatant, clarify it as before and add it to the one collected previously.
5) Add 1 volume of Viro-PEG for 4 volumes of viral suspension
6) Mix well during 60s
7) Refrigerate 4H to Overnight under constant rocking – recommended overnight
8) Centrifuge 3000xg x 60 min at 4°C
9) Remove supernatant
10) Resuspend the pellet in 1/10th to 1/100th of the original volume.

2.8. Titering Lentiviral production using a fluorescent reporter plasmid

Once collected and/or concentrated, functional viral titer of a fluorescent reporter lentivirus can be determined by flow cytometry.

1) Prepare a **1/100th** viral dilution in complete culture medium

2) Trypsine HEK293T and plate **200 000** cells/well in a 6-well plate, in **2 mL** complete culture medium.
3) Do not allow cells to adhere.
4) Add **100 µL**, **50 µL**, **10 µL**, **5 µL**, **2 µL** and **1 µL** of the viral dilution to the cells
5) Mix thoroughly but gently and incubate at 37°C.
6) Incubate for 72H
7) Wash the cell twice with 1X PBS to eliminate leftover virus in the medium
8) Trypsinize the cells, fix them and proceed to flow cytometry analyses
9) Determine the percentage of fluorescently labelled cells
10) Note the dilution for % comprised between 0 and 10%
11) Calculate biological titer according to the following formula:

Biological titer = % x (N / V) x D

% = percentage of fluorescent cells – should be comprised between 0 and 10%
N = number of cells at the time of transduction (here = 200.000)
V = volume of dilution added to obtain the %, in mL
D = dilution factor (100)

3. Quality controls

The following *in vitro* assays are conducted to qualify function, quality and performance activity of each batch of HYVIR produced.

Specification	Standard Quality Controls
Purity	Silica Gel TLC assays. Every compound shall have a single spot.
Sterility	Thioglycolate assay. Absence of fungal and bacterial contamination shall be obtained for 14 days.
Biological activity	Every lot shall have an acceptance specification of >80% of the activity of the reference lot.

4. Troubleshooting

Our dedicated and specialized technical support group will be please to answer any of your requests and to help you with your transfection experiments. You can contact them at tech@ozbiosciences.com. In addition, do not hesitate to visit our website www.ozbiosciences.com for updated information.

5. Related products

- **Mag4C-Lv Kit** for capturing, concentrating and storing of Lentivirus.
- **Viro-PEG Lentivirus Concentrator** for the capture and concentration of lentiviral particles, providing an easy and straightforward method to efficiently concentrate lentiviral particles without using ultracentrifugation.
- **LentiBlast Premium Transduction Enhancer** Improve your transduction protocol for stem and CAR-T cell therapy.
- **ViroMag** for enhancing viral transduction efficiency (suitable for all viruses).

Our technical support will be pleased to answer any of your request and to assist you in your experiments. Do not hesitate to contact us for all complementary information and remember to visit our website in order to stay inform on our latest breakthrough technologies and update: http://www.ozbiosciences.com

Purchaser Notification

Limited License

The purchase price paid for HYVIR by end users grants them a non-transferable, non-exclusive license to use the kit and/or its separate and included components (as listed in the section 1, Kit Contents). These reagents are intended **for internal research only** by the buyer. Such use is limited to the use in the product manual. Furthermore, research only use means that this kit and all of its contents are excluded, without limitation, from resale, repackaging, or use for the making or selling of any commercial product or service without the written approval of OZ Biosciences.
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Product Use Limitations

The HYVIR™ is developed, designed, intended, and sold for research use only. It is not to be used for human diagnostic or included/used in any drug intended for human use. All care and attention should be exercised in the handling of the kit components by following appropriate research lab practices. For more information, or for any comments on the terms and conditions of this License, please contact:

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