TIPS & TRICKS FOR SUCCESSFUL LENTIVIRAL TRANSDUCTION

OZBIOSCIENCES
The art of delivery systems

Cells transduction represents a powerful tool to genetically modify a wide variety of cell lines and primary cells. Unfortunately, working with Lentivirus can be challenging. To identify and overcome pitfalls, several parameters can be considered. This application note describes the practices to follow for maximizing your lentiviral transduction efficiency.

VIRUS PRODUCTION

Packaging Cells

Cell culture conditions for each type of cells

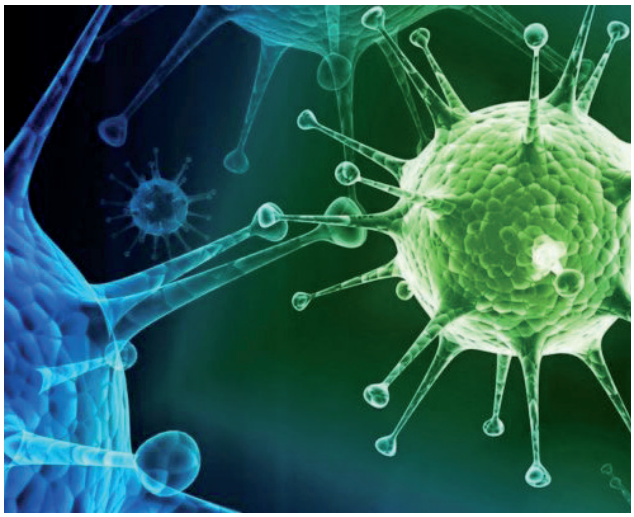
The production of viral particles depends greatly on the health of both the packaging cells and the target cells.
It is important to maintain cells in good conditions and to cultivate them correctly in their respective environments. A specific complete culture medium and specific culture vessel must be used depending on what type of cell is being used. Before beginning, learn about your cells and find their growing rate, concentration for seeding, optimal density or concentration at the time of experiment, and time for doubling population.
The more you know about your cells, the happier they are.
HEK-293T are one of the most used cells for virus production. This fast growing cell line needs to be regularly passaged (when reaching 70-80% confluence) and seeded the day before transfection so that they reach the ideal confluence at the time of transfection (50-60% confluence) or prepared the time of infection (70-80% confluence).

Absence of contaminant and mycoplasma free

Efficient and reproducible viral production is guaranteed by healthy cells and can be dramatically altered by the presence of mycoplasma or contaminants. Mycoplasmas compete with host cells for nutrients and persist undetected until charge is high enough to be visible in the culture medium. They are thus hard to detect and they impact transfected or infected cells in several ways leading to modified DNA or RNA synthesis, lower proteins levels or altered cellular metabolism, receptor, ion channels or growth factors expression.

Decrease in growth rate or loss of adherence as well as loss of contact inhibition when reaching confluence should be seen as a precursor to the presence of mycoplasma.

Check your cell cultures on a regular basis to ensure working with cells in good health to get an optimal production of viral particles.



The importance of the transfection reagent

Several solutions exist on the market such as CaPO Kit or common transfection reagents but efficiency is either not optimal or lacks reproducibility. Moreover since the emergence of 3rd generation viral vectors, it is now a challenge to deliver 4 plasmids in the same cell at the same time while respecting the stoichiometry of each one with high efficiency to produce a good quality virus with high titer.
Only the latest generation of transfection reagents are able to package these 4 plasmids of different sizes to protect them from extracellular degradation and to deliver them into the cells.

Helix-IN™ DNA transfection reagent, a new patented polymer-based reagent, opens up new possibilities for addressing issues of classical transfection technologies.

This reagent is able to co-transfect multiple plasmids at the same time. Moreover its stealth properties lower detection by cell sensors so that more DNA is delivered to the nucleus leading to an enhanced viral production.

How to obtain a maximal efficiency in transfection using Helix-IN™

- Prepare a mix of the transfer plasmid and packaging plasmids respecting the specific required amounts of each ones in serum-free medium
- Add transfection reagent at the recommended ratio
- Incubate for 30 mins at room temperature to form complexes
- Add the complexes onto the cells in a drop-wise manner and gently rock the plate back and forth to ensure a correct distribution of the complexes
- Optionally, medium can be changed 6h after transfection.

NOTE

It is important to refer to the specific protocol of your transfection reagent and to keep in mind that, depending on many parameters such as cell type, culture conditions etc... it may be necessary to optimize the conditions of transfection (ratios, DNA quantitty etc...).

VIRAL PARAMETERS

Check your Viral Production

Virus Titration

When we talk about viral titer we refer to Transduction Unit (TU) that represents only complete and active viral particles. As opposed to physical particles (PP) that contain any type of particles including empty and inefficient transducing ones, TU refers to a non-overestimated titer reflecting the capacity of the viral production to infect and transduce target cells. More importantly the difference between physical particles and transduction unit may vary from a ratio PP/TU of 100 to 1000.

Check out the following protocol in order to calculate your viral titer according to the method by Tiscornia *et al* (Tiscornia G., Nat Protoc. 2006;1(1):241-5.).

Rapid protocol for lentiviral titration

- Prepare a tenfold serial dilution of viral supernatant
- Prepare the cells at the time of infection:
  - Treat plate with Poly-L-Lysine to enhance cell adherence to substrate (500µL per well x 15 min at RT)
  - Remove liquid and proceed immediately
  - Add 100.000 cells per well in a 24-well plate in a final volume of 500 µL (do not allow the cells to adhere to substrate)
- Add 20 µL of each viral dilution to the cells, mix thoroughly and incubate at 37°C for 48h
- Determine the % of infected cells by flow cytometry
- Calculate biological titer (transducing units / ML) according to the following formula:

Biological titer (TU/mL) = F x (C / V) x D

With:
F : % of infected cells (between 0-20 %) / 100
C : cell number at the time of infection
V : volume of virus expressed in mL
D : dilution factor used to obtain an infection rate between 0 and 20%

Ensuring Quality (Harvesting/Clarification)

Taking care of the cells and choosing an optimal transfection reagent always leads to an increased viral production.
However once produced in the supernatants, virus needs also special attention and final titer will depend on some important parameters:

Viral collection can be done at a single point after transfection or from multiple time points.
The advantage of multiple harvests approach is that fresh medium is used to replaced consumed and acidified old one; the main limitation being that final viral solution is diluted compare to single time collection (*refer to the following section «Concentrate viral production»*) and should thus be used when concentration procedure is forecasted.

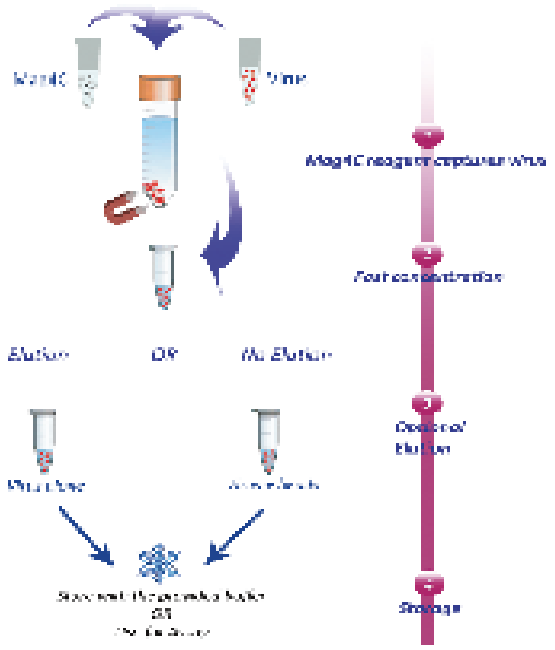
Once produced, viral particles are released into the cell supernatant where "contaminants" such as exosomes, cell membrane debris or apoptotic bodies can impair the overall production.
For this reason it is important, if not essential, to clear the viral particles. Generally a centrifugation step at 3500rpm x 15min x 4°C or filtering the supernatant over 0.45µM membrane is sufficient enough to isolate viral particles from unwanted solution.

NOTE

During transient transfection, there is a peak of vector particle production on days 2 and 3 post-transfection that drops significantly after day 4.
Considering concentration procedure, the supernatant should be collected after 24 and 48h ; viral suspension harvested at day 1 should be clarified, and kept at 4°C until it is pooled and further concentrated with day 2 suspension. If the particles are to be used without concentration, prefer collect the viral suspension 48 to 72h post-transfection.

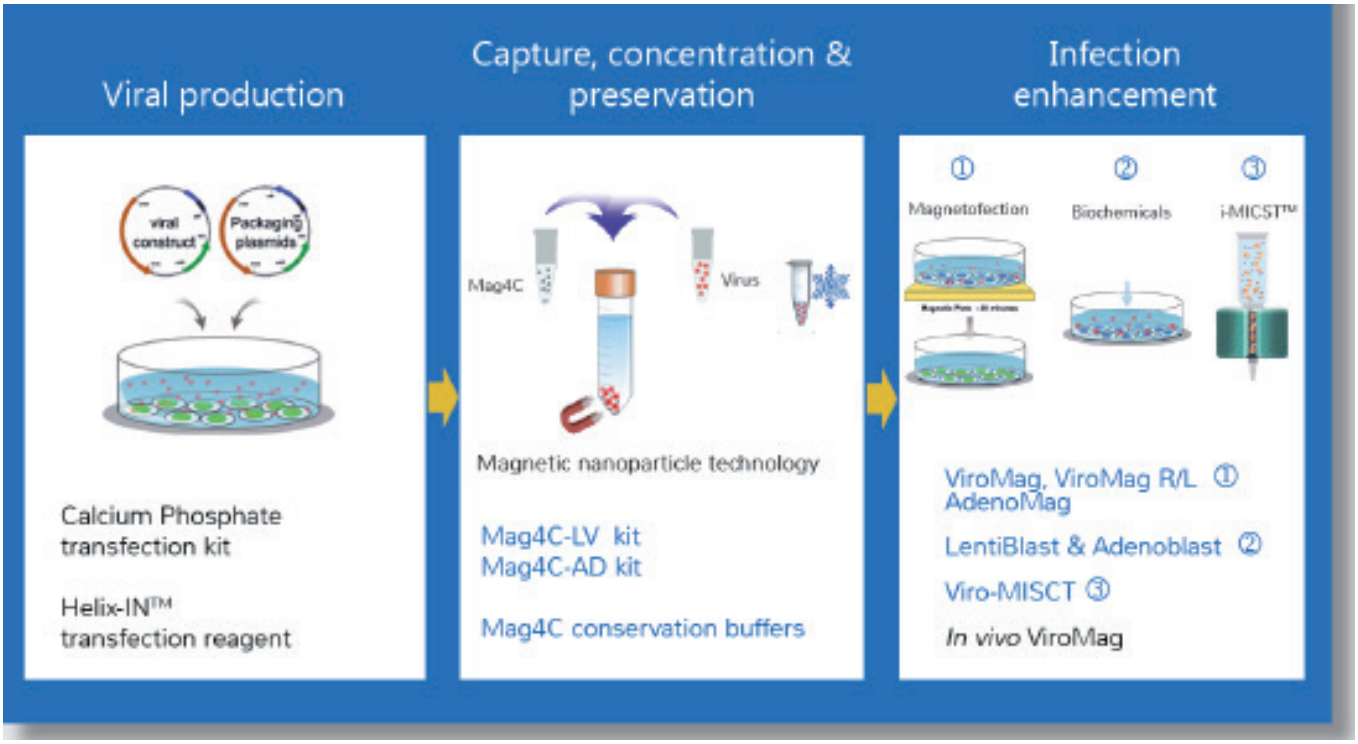
Concentrate Viral Production

Depending on the harvesting procedure (single time or multiple times – *refer to the paragraph «Ensuring Quality»*) or on the biological titer, it may be necessary to concentrate the viral particles.
To achieve this, OZ Biosciences provides two different methods, a kit using chemical properties (*Viro-PEG Lentivirus Concentrator*) and a reagent based on the Magnetofection technology (*Mag4C-LV*).



Mag4C-LV Protocol

These two methods allow concentrating Lentiviruses up to 100 times without impairing the infectious capacities of the viral particles.
Viro-PEG is more adapted for scaling up processes while *Mag4C-LV*, using magnetic nanoparticles, allows to capture and concentrate lentiviral particles in culture medium through electrostatic and hydrophobic interactions with 80-99% efficiency. *Mag4C-LV* allows a rapid concentration of viral particles with minimized hazardous handling avoiding ultracentrifugation, precipitation and chemical steps. Once captured and concentrated, the viral particles can be kept bound to the magnetic beads and used for Magnetofection-based transduction assays, or eluted from the beads and/or stored.



OZ Biosciences has created a comprehensive line of reagents dedicated to viral applications

VIRAL PARAMETERS

Optimize storage and thawing conditions

After production and collection, supernatants need to be clarified in order to remove most of the contaminants (cellular debris) and concentrated or not. It is then recommended to aliquot lentiviral vectors in smaller volumes that will be used as working solutions; aliquots must be stored at -80°C and never in liquid nitrogen.
Avoid repeated freeze/thawing cycles that impair the integrity of the viral particles resulting in a dramatic loss of biological titer.
Prefer keeping the virus thawed at +4°C for a short period of time rather than returning it to -80°C; lentivirus at +4°C will be stable for several days (discard after 1 week).

In order to extend the life of viral particles stored at -80°C and to lower the loss of infectivity due to repeated freezing/thawing cycles, OZ Biosciences has developed a conservation buffer aimed at protecting viral particles from degradation during these episodes (*Mag4C-LV Conservation Buffer*).

Optimize the vector

3 generations of lentiviral vectors are routinely used (even if the 1st one is now outdated and the 2nd one tends to disappear). They are composed of various vector sequence elements that can have a great impact on packaging and thus on the final viral titer.
Beside the number of plasmids needed, respectively 3 and 4, the main difference between 2nd and 3rd generation is the introduction of a deletion in the U3 region of the 3' LTR and the absence of Tat in the latter one.

Various 5' LTR sequences, promoters and Post-Transcriptional Regulatory Elements such as WPRE can be used and each element could drive differences in the transgene expression into the host cells.
Common to all type of generations, the lentiviral transfer plasmid encoding your insert of interest also has to be optimized as the sequence between and including the LTR is integrated into the host genome upon viral transduction and should not span over 10 kb to ensure a maximal efficiency.

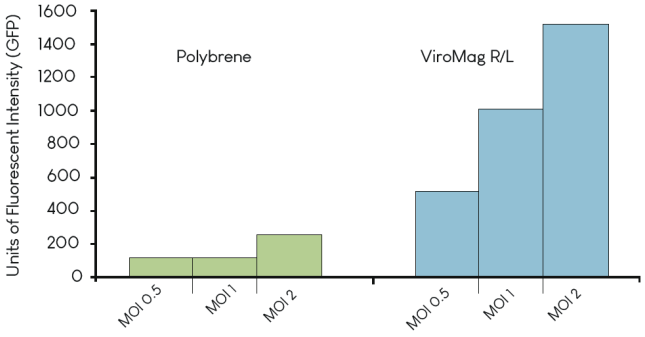
TRANSDUCTION ENHANCERS

Improve transduction with enhancers

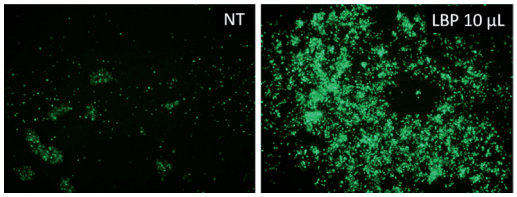
Once produced and titrated, the virus is used to infect and transduce the target cells. One of the most challenging points is to rapidly put cell and virus into contact; enhancing cell-virus interactions is the key to an efficient transduction process. For a while some solutions such as Polybrene have been around but not without severe drawbacks such as of toxicity.
In order to propose a safe method to improve transduction, OZ Biosciences has developed the Magnetofection technology which induces a rapid concentration of viral particles under the infiuence of a magnetic field.
Using *ViroMag RL* reagent, almost all of the viral load is put into contact with the cell surface within minutes without altering the cell membrane integrity.

Recently, *LentiBlast Premium*, a chemical-based transduction enhancer issued from OZ biosciences' patented technology, has proven a great success.
Users report that this reagent is non-toxic and enhance lentiviral transduction efficiencies in a large variety of cells but particularly in CD34+ hematopoietic stem cells & T lymphocytes - making it ideal to improve their transduction protocols for stem and CAR-T cell therapies.

ViroMag RL is highly efficient for lentiviral infection on U87-CD4$^+$ cells



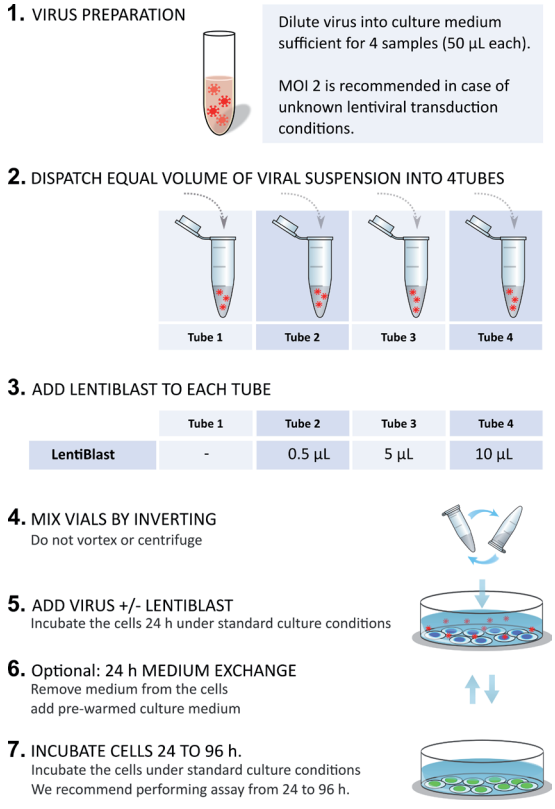
This new formulation neutralizes electrostatic repulsions between membrane and viral particles. As a result it enhances viral fusion with cell membrane.



Primary CD34+ transduced with Lentivirus encoding for GFP at low MOI of 5 in presence (or not) of LentiBlast Premium (LBP). After 72 h incubation, transduction efficiency was visualized.

Optimize transduction enhancers efficiency

Following the standard protocols is the best option to improve infection and transduction. However, depending on many parameters such as cell type, culture conditions, lentivirus (generation, ratio active/non active particles, transfer gene...) it may be necessary to optimize the conditions of transduction. Whichever enhancer is used (*ViroMag RL or LentiBlast Premium*), we generally recommend (1) to use a fixed MOI with ranging doses of enhancers and (2) to fix the amount of enhancers previously found and to vary the MOI.



LentiBlast Premium Quick Protocol

MORE PARAMETERS

Maximize transduction

Beside enhancer optimization, it is still possible to maximize infection and transduction by playing on several parameters such as incubation time, infection procedure or cell culture.

- Incubation time

Depending on the viral vector, the transfer plasmid and thus on the kinetics of expression, time course before visualizing infection or transduction experiment may vary a lot. However analyzing experiment after at least 72 hours appears a good choice as analysis at this time point also minimizes the contribution of false positive signals due to pseudotransduction.
For example, when using a reporter gene expressing green fluorescence protein (GFP), it is recommended to determine transduction efficiency by flow cytometry after 72h to ensure that all transduced cells are expressing GFP at detectable levels.

- Infection procedure

Whatever the cell culture phenotype is (suspension or adherent), a spinoculation step can facilitate the transduction process although it may decrease the survival sensitive cells such as T cells (spinoculation is a quite long process where infection occurs during a 2h x 2500 rpm centrifugation step). As an alternative, once viral particles are mixed with *LentiBlast Premium*, a classic centrifugation step can be performed to enhance the contact between virus and cell surface. These steps are not at all mandatory when using Magnetofection technology and *ViroMag RL* reagent due to the action of the magnetic field that attracts viral particles toward the cellular membrane ; mimicking centrifugation process.

- Reverse transduction

This method refers to a strategy where viral vectors are added to the culture plate first so that the cells get transduced during attachment. This is a good alternative to classic infection protocol as overall efficiency can be improved.

- Cell culture

Depending on cell type and the viral strain, the serum part of the culture medium can be modified at the time of transduction: either cells can be infected in total absence of serum or in presence of serum from another species.

Check vital cellular parameters following transduction

Concomitantly to the measurement of transgene expression, it may be also essential to follow the correct development of the infected/transduced cells.
Some parameters such as proliferation, protein content or cellular stress/activation can be monitor using different assays kits (MTT, WST-8, Rezasurin, Bradford, Fluoprodige, ROS and NOS measurement...).

For more information watch our video online!

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rev 05/2024