Development and Optimization of a High Titer Recombinant Lentivirus System

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To enable simple and effective high titer recombinant lentivirus production, we examined key parameters for the generation of lentivirus including: transfection conditions, DNA vector selection, media change, and incubation time. Adherent or suspension adapted 293-derived cells can be used as production hosts. These results illustrate the importance of optimizing transfection processes for high titer recombinant lentivirus production.

Introduction

Lentivirus is an enveloped single-stranded RNA virus from the *Retroviridae* family. Its ability to infect dividing and non-dividing cells has led to the widespread utilization of recombinant lentivirus as a gene delivery vehicle. In addition, lentivirus has an efficient integration mechanism which leads to robust and stable transgene expression in target cells.

Recombinant lentivirus production is frequently accomplished in human embryonic kidney derived cells that stably express the SV40 large T antigen (e.g. HEK 293T/17 cells). These cells are transiently transfected with three or four plasmids that encode the gene of interest (GOI), the vesicular stomatitis virus envelope protein G (VSV-G), and the essential viral genes: *gag*, *pol*, and *rev*. Once all the genes are expressed at sufficient levels the virus assembles and buds through the plasma membrane to form an enveloped virion pseudotyped with VSV-G which conveys the ability to transduce a broad range of mammalian cell types. The virus-containing supernatant can be collected and filtered to remove any cells. Target cells are transduced with the recombinant lentivirus in the presence of hexadimethrine bromide or *Transduce*IT™ Transduction Reagent to increase virus aggregation and cellular uptake.

Cell Confluency

A key parameter for successful transfection and subsequent virus production is the cell confluency at the time of transfection. Low confluency can lead to cytotoxicity and lower transfection efficiency. Performing transfections at high confluence, e.g. 85-90%, will maximize the protein expression levels and subsequent titers.

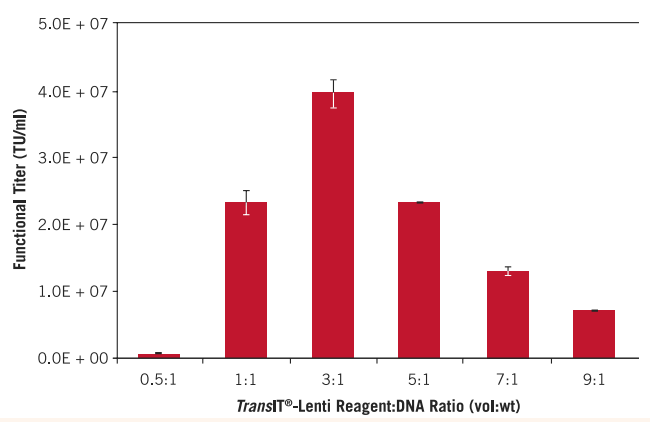

Figure 1. Optimization of reagent-to-DNA ratios. Adherent 293T/17 cells were transfected in a 12-well plate format using the vectors pLKO.1-puro-CMV-TurboGFP™ (MilliporeSigma) transfer vector and the Lentivirus Packaging Mix, Powered by MISSION® Genomics (Mirus Bio) with the *Trans*IT®-Lenti Transfection Reagent (Mirus Bio) at varying reagent-to-DNA ratios (0.5:1 to 9:1, 1 µg total DNA per well). The supernatant was harvested, filtered (0.45 µm), and titered using 293T/17 cells. Lentivirus transductions were performed in the presence of 8 µg/ml *Transduce*IT™ (Mirus Bio) and GFP expression was measured 72 hours post-transduction using a Guava® easyCyte™ 5HT Flow Cytometer (MilliporeSigma). Error bars represent the range of duplicate wells. Functional titers were calculated using virus dilutions with less than 20% GFP positive cells.

Reagent-to-DNA Ratio

The transfection reagent-to-DNA ratio is a critical parameter for efficient nucleic acid delivery. The positively charged transfection reagent must be supplied in sufficient quantities to effectively condense and coat the negatively charged plasmid DNA. For ease of use, we formulated the *Trans*IT®-Lenti Transfection Reagent to perform optimally at a 3:1 reagent-to-DNA ratio (volume:weight). Deviating from the optimal reagent-to-DNA ratio can adversely affect the virus titers (Figure 1). Under these conditions, the total plasmid concentration of 1 µg/ml of culture resulted in maximal lentivirus titers. Depending on the potency of the vector system, varying the total DNA concentration while maintaining the reagent-to-DNA ratio may increase lentivirus titers.

The order of addition, composition and stoichiometry of the packaging and transfer plasmids can greatly influence the titers obtained. Depending on the generation of the packaging system, three or four plasmids need to be premixed prior the DNA being added to the transfection reagent to ensure that the transfection complexes are not formed preferentially with one plasmid over another. In addition, if using individual packaging plasmids we recommend a starting ratio of 6:4:1:1 of transfer:gag-pol:rev:VSV-G vectors. If using a lentivirus packaging premixture we recommend an initial ratio of 1:1 between the packaging mixture and the transfer plasmid.

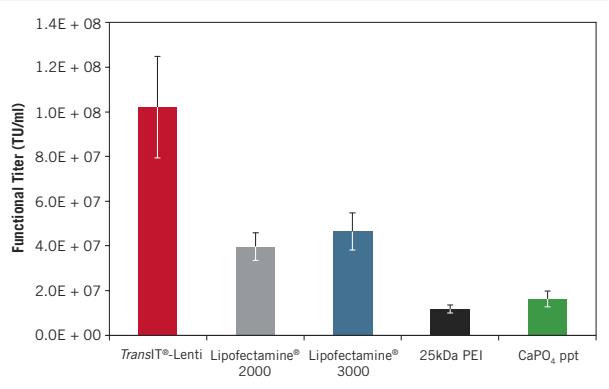

Figure 2. High Functional Titers with the *Trans*IT®-Lenti Transfection Reagent. Adherent 293T/17 cells were transfected in a 6-well plate with pLKO.1-puro-CMV-TurboGFP™ (MilliporeSigma) transfer vector and the Lentivirus Packaging Mix Powered by MISSION® Genomics (1:1 ratio, 2 µg/well, Mirus Bio) with the following reagents: *Trans*IT®-Lenti (3:1, vol:wt, Mirus Bio), Lipofectamine® 2000 (3:1, Thermo Fisher Scientific), Lipofectamine® 3000 (3:1:1, vol:vol:wt, Thermo Fisher Scientific), 25 kDa PEI (6:1, Polysciences), or $CaPO^4$ precipitation (4 µg pDNA/well, GE Life Sciences). The supernatant was harvested, filtered (0.45 µm), and titered using 293T/17 cells. Lentivirus transductions were performed in the presence of 8 µg/ml *Transduce*IT™ (Mirus Bio) and GFP expression was measured 72 hours post-transduction using a Guava® easyCyte™ 5HT Flow Cytometer (MilliporeSigma). Error bars represent the standard deviation of triplicate transfection complexes titered individually. Functional titers were calculated using virus dilutions with less than 20% GFP positive cells.

Transfection Reagent Selection

High efficiency transfection is necessary, but not sufficient for high titer recombinant lentivirus production. To empirically determine the best transfection formulation for lentivirus production we initially screened our compound libraries using a reporter assay system; however, when we compared the reporter assay output with functional lentivirus production we did not always observe a correlation. Therefore, we employed a targeted compound screening approach to directly test the effect of reagent formulation on functional lentivirus titers. During this process we identified a novel formulation, the *Trans*IT®-Lenti Transfection Reagent, which yields up to 8-fold higher functional titers in head-to-head comparisons with other commonly used transfection technologies in 293T/17 cells (Figure 2).

Plasmid DNA

Vector design is a critical factor in recombinant lentivirus production. Advances in the safety of lentivirus production have been accomplished through the deletion of non-essential components of the virus genome, in addition to, separation of essential elements onto different plasmid vectors. Furthermore, the composition and design of the plasmid vector greatly affects the overall virus titer.

Within a given laboratory, a single lentivirus packaging system is often paired with a variety of individual transfer vectors. Therefore, we tested the degree to which lentivirus titers were altered by combining different transfer vectors with a single packaging system. In studying three different commercially available third generation transfer vectors we observed up to a 4-fold difference in lentivirus titers (Figure 3A). When performing system-to-system comparisons (i.e. when all key components are varied including: transfer vectors, packaging mix and transfection reagent), the results are compounded and a combined 100-fold difference is observed (Figure 3B). This illustrates the synergy between the essential components of recombinant lentivirus production.

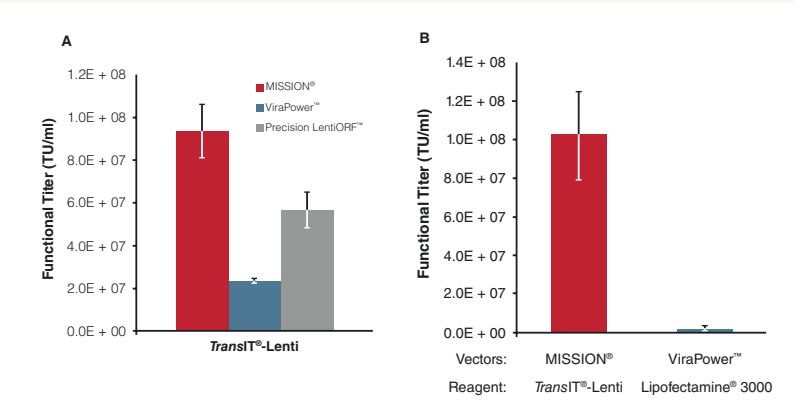

Figure 3. Vector selection greatly affects lentivirus titer. (A) Adherent 293T/17 cells were transfected in a 6-well plate with one of three transfer vectors: MISSION® pLKO.1-puro-CMV-TurboGFP™ (MilliporeSigma), ViraPower™ pLenti6.2-GW/EmGFP (Thermo Fisher Scientific), or Precision Lenti-ORF™ RFP (GE Life Sciences), and the Lentivirus Packaging Mix Powered by MISSION® Genomics (total 2 µg/well, MirusBio) using the *Trans*IT®-Lenti Transfection Reagent (3:1, vol:wt, Mirus Bio). **(B)** System-to-system comparisons were performed using either MISSION® vectors pLKO.1-puro-CMV-TurboGFP™ and Lentiviral Packaging Mix (MilliporeSigma) and the *Trans*IT®-Lenti Transfection Reagent (MirusBio) or ViraPower™ vectors (pLenti6.2-GW/EmGFP and Lentiviral Packaging Mix (Thermo Fisher Scientific) and the Lipofectamine® 3000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturers' protocol (2µg/well). The supernatant was harvested, filtered (0.45 µm), and titered using 293T/17 cells. Lentivirus transductions were performed in the presence of 8 µg/ml *Transduce*IT™ (Mirus Bio) and GFP was measured 72 hours post-transduction using a Guava® easyCyte™ 5HT Flow Cytometer (Millipore Sigma). Error bars represent the standard deviation of triplicate transfection complexes titered individually. Functional titers were calculated using virus dilutions with less than 20% GFP positive cells.

Media Change Post-transfection

The harvest procedure during recombinant lentivirus production often includes a media change 24 hours post-transfection, as well as, multiple harvests at 48 and 72 hours post-transfection. To investigate the importance of these parameters we tested functional lentivirus titers in media samples post-transfection, as well as, undisturbed wells at 48 and 72 hours post-transfection. By measuring the functional titers obtained from the media samples we found that the majority of the recombinant lentivirus was produced between 24-48 hours post-transfection (Figure 4). We also observed no difference in functional lentivirus titers when the virus was harvested at 48 or 72 hours post-transfection without a media change suggesting that this step can be omitted

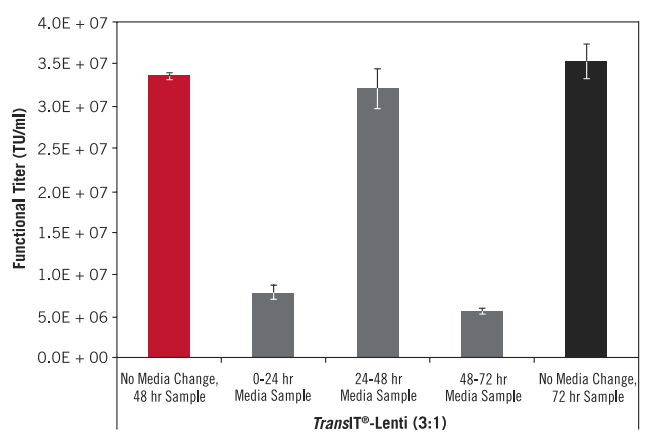

Figure 4. Single Harvest and No Media Change Required. Adherent 293T/17 cells were transfected in a 6-well plate with pLKO.1-puro-CMV-TurboGFP™ (MilliporeSigma) transfer vector and the Lentivirus Packaging Mix Powered by MISSION® Genomics (1:1 ratio, 2 µg/well, Mirus Bio) with the *Trans*IT®-Lenti Transfection Reagent (Mirus Bio) at a 3:1 reagent-to-DNA ratio (vol:wt), The supernatant was harvested at the indicated time points, filtered (0.45 µm), and titered using 293T/17 cells. Lentivirus transductions were performed in the presence of 8 µg/ml *Transduce*IT™ (Mirus Bio) and GFP expression was measured 72 hours post-transduction using a Guava® easyCyte™ 5HT Flow Cytometer (MilliporeSigma). Error bars represent the standard range of duplicate wells titered individually. Functional titers were calculated using virus dilutions with less than 20% GFP positive cells.

without adverse affects. These findings demonstrate a workflow that reduces experimental complexity and workload.

Cell Type and Growth Characteristics

Adherent 293T cells are commonly used for recombinant lentivirus production. However, when larger quantities of virus are required or when a chemically defined system that lacks animal-derived components is needed, it is advantageous to employ suspension adapted 293 cell types. Transient transfection efficiencies can vary widely in different serum-free media formulations possibly due to charged components interfering with the electrostatic complex formation between the transfection reagent and the plasmid DNA. For a suspension cell platform, we have found that Thermo Fisher Scientific Freestyle™ 293-F cells grown in FreeStyle™ F17 Expression Medium, supplemented with 0.2% poloxamer 188 is amendable to single cell growth and high titer lentivirus production using a standard transfection protocol. In head-to-head comparisons with commonly used transfection technologies the *Trans*IT®-Lenti Transfection Reagent yields the highest titers (Figure 5).

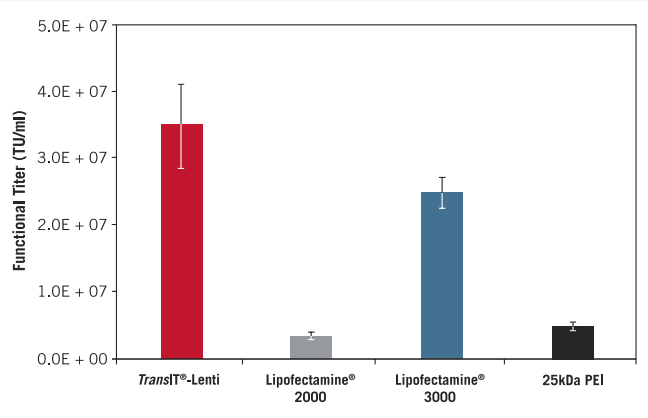

Figure 5. Suspension 293 cells support lentivirus production. Suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium were transfected with pLKO.1-puro-CMV-TurboGFP™ (MilliporeSigma) transfer vector and the Lentivirus Packaging Mix powered by MISSION® Genomics (1:1 ratio, 1 µg/ml, Mirus Bio) with the following reagents: *Trans*IT®-Lenti (3:1, vol:wt), Lipofectamine® 2000 (2:1, Thermo Fisher Scientific), Lipofectamine® 3000 (2:2:1, Thermo Fisher Scientific) or 25 kDa PEI (4:1, Polysciences). The supernatant was harvested, filtered (0.45 µm), and titered using 293T/17 cells. Lentivirus transductions were performed in the presence of 8 µg/ml *Transduce*IT™ and GFP expression was measured 72 hours post-transduction using a Guava® easyCyte™ 5HT Flow Cytometer. Functional titers were measured from virus dilutions with less than 20% GFP positive cells. The error bars represent the standard deviation of triplicate wells.

Conclusions

We systematically optimized the experimental variables surrounding recombinant lentivirus production including: cell confluency, transfection reagent-to-DNA ratio, reagent formulation, DNA vectors, media change, and incubation time. The highest titers are achieved when 293T/17 cells are transfected at 90% confluency using the *Trans*IT®-Lenti Transfection Reagent at a 3:1 reagent-to-DNA ratio (vol:wt). Using these conditions, a media change is not required post-transfection and a single harvest at 48 hours post-transfection offers convenience and less handling of the cells. In addition, the transfer and packaging plasmid also play a critical role in the titers achieved. The combination of transfection reagent, transfer vector and packaging premix can lead to a 100-fold difference in titers in head-to-head comparisons. The *Trans*IT®-Lenti Transfection Reagent also supports recombinant lentivirus production in suspension 293-derived cell types. The *Trans*IT®-Lenti Transfection System combining Mirus' novel transfection formulation and the Lentivirus Packaging Mix Powered by MISSION® Genomics provides exceptional lentivirus titers, increased flexibility and reduced workload.

For more information about the *Trans*IT®-Lenti Transfection Reagent or the Lentivirus Packaging Mix Powered by MISSION® Genomics visit www.mirusbio.com/transit-lenti

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