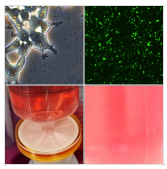

Version 3

Apr 24, 2021

Efficient third generation lentiviral particle production V.3

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bugxntxn

Neville Ng

ABSTRACT

The overexpression of a gene of interest by third generation lentiviral particle generation systems is a critical process in molecular biology, cell biology and gene therapy research. While many lentiviral protocol production methods have been discussed in literature, this protocol takes into account previously established optimisations with the aim to minimise user handling time, cost, and maximise practical yield. This protocol allows for at least 6 days of consecutive viral particle collection without compromising HEK293T cell culture or viral production efficiency, and can be easily and cost effectively reproduced in basic cell culture laboratories.

DOI

dx.doi.org/10.17504/protocols.io.bugxntxn

PROTOCOL CITATION

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WHAT'S NEW

Minimal cost and user handling time, compatible with animal-free/serum-free cell culture and includes cryopreservant in viral particle aliquots to maintain transduction efficiency through multiple freeze thaw cycles.

KEYWORDS

lentiviral, HEK293T, differentiation, lentivirus, overexpression, plasmid, viral particle, production

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MATERIALS TEXT

Cell culture

HEK293T cell culture
FreeStyle™ 293 Expression Medium (Thermo Fisher Scientific #12338018) OR
DMEM/F12 (Thermo Fisher Scientific #21331020) + GlutaMAX™ Supplement (Thermo Fisher Scientific #35050061) + generic fetal calf serum OR Gibco KnockOut™ Serum Replacement (Thermo Fisher Scientific #10828028)
Gibco Penicillin-Streptomycin (Thermo Fisher Scientific #15140122)

Transfection

Linear 10-20 kDa PEI (Sigma #765090 or Polysciences #23966-1)
Plasmid(s) containing gene of interest

Plasticware

Generic tissue culture 75 cm2 cell culture vessels and 10 mL serological pipettes
Generic round bottom ultracentrifuge tubes ~35 mL
Generic sterile 50 mL centrifuge tubes
Generic 50 mL syringe and 0.45 µm pore PES filters

SAFETY WARNINGS

All stages should be performed with appropriate safety precautions specific to local standards. Lentivirus particles can be inactivated by hypochlorite, peroxide and ethanol based sterilisation agents.

Lentiviral transfection

1 Maintain HEK293T cells in 0.15 mL/cm^2 (e.g. 12 mL in 75 cm^2 flask) animal product free FreeStyle 293 Expression Medium, foetal serum free DMEM/F12 + 2 mM GlutaMAX + 5% Knock Out Serum Replacement or DMEM/F12 + 2 mM GlutaMAX + 5% FBS. Include 50 U/mL Penicillin and Streptomycin to reduce risk of bacterial contamination if necessary.

Dissociate and subculture HEK293T to ~70% confluency per virus of interest.

Viral particle production can be performed within at least 15 passages without loss of yield.

> Ausubel LJ, Hall C, Sharma A, Shakeley R, Lopez P, Quezada V, Couture S, Laderman K, McMahon R, Huang P, Hsu D, Couture L (2012). Production of CGMP-Grade Lentiviral Vectors.. BioProcess international.

2 For each lentiviral plasmid containing gene of interest and packaging plasmid vectors, calculate reagent volumes required for 12 µg gene of interest vector, 6 µg pMDLg/pRRE (Addgene #12251), 3 µg pRSV-Rev (Addgene #12253) and 3 µg pCMV-VSV-G (Addgene #8454) and 60 µg PEI per 75 cm^2 of HEK293T culture.

Maximal transfection efficiency by PEI complex is typically obtained with higher proportions of PEI:DNA (Figure 1). We observed no differences in transfection with KSR or FBS -based medium.

Citation: Michelle Newbery, Simon Maksour, Amy Hulme, Neville S Ng, Mirella Dottori, Lezanne Ooi (04/24/2021). Efficient third generation lentiviral particle production. https://dx.doi.org/10.17504/protocols.io.bugxntxn

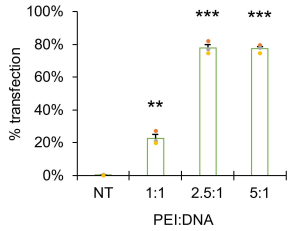

Figure 1. Approximately 2.5-5:1 PEI:DNA provides maximal transfection efficiency. Experiment performed with a hNGN2-eGFP containing plasmid (n = 3, error presented as SEM; ** p < 0.01, *** p < 0.001, analysis by 1-way ANOVA and Holm-Sidak post-hoc multiple comparisons test).

5m

3 Prepare PEI-DNA solution in 1.5 mL of DMEM/F12 and incubate for **00:05:00** at ambient temperature.

4 Add PEI-DNA complex solution to cell culture flask(s) and return to incubator **Overnight** .

Note PEI-DNA complex transfection can occur without usage of low serum transfection medium products.

PEI-DNA complex precipitate should be microscopically visible as ~0.3 µm objects (Figure 2).

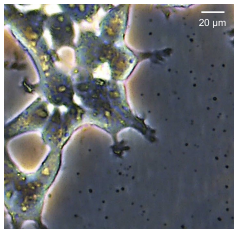

Figure 2. Phase contrast image of HEK293T cell culture with PEI:DNA complex particles.

González-Domínguez I, Grimaldi N, Cervera L, Ventosa N, Gòdia F (2019). Impact of physicochemical properties of DNA/PEI complexes on transient transfection of mammalian cells.. New biotechnology.
https://doi.org/10.1016/j.nbt.2018.09.005

5 Each day collect medium in an appropriate sized sterile container (e.g. 50 mL centrifuge tubes or 120 mL specimen collection tubes), and store at **4 °C** . Proceed to viral particle concentration at selected endpoint (Step 6).

We utilise viral particle concentration to avoid prolonged incubation of pluripotent or multipotent stem cells in HEK293T culture medium during viral transduction experiments. If viral particle concentration is not necessary, freeze immediately at **-80 °C** .

Note a single collection step is performed on day 6 due to negligible difference in loss of yield between day 3 and 6, which avoids multiple rounds of centrifugation.

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Rahman, H.; Taylor, Josephine; Clack, Beatrice; Stewart, R. S.; and Canterberry, Sarah C (2013). Effects of Storage Conditions on the Morphology and Titer of Lentiviral Vectors. SFA ScholarWorks.

Viral particle collection can be performed for at least up to 7 days.

Ichim CV, Wells RA (2011). Generation of high-titer viral preparations by concentration using successive rounds of ultracentrifugation.. Journal of translational medicine. https://doi.org/10.1186/1479-5876-9-137

Lentiviral particle concentration 1h 33m

6 Sterilise ultracentrifuge tubes and appropriate sized viral particle aliquot tubes (0.2 or 1.5 mL microcentrifuge tubes).

For each 75 cm^2 flask if collecting 12 mL over 6 days, at least 2 x 36 mL ultracentrifuge tubes are required.

7 Centrifuge pooled viral particle supernatant at **300 x g, 00:03:00** to remove cell debris. 3m

8 Filter supernatant from each tube through a 0.45 µm pore PES filter into corresponding ultracentrifuge tubes.

9 Balance ultracentrifuge tubes within 0.1 g by weighing and adding appropriate amount of medium.

10 Collect viral particles by centrifugation of filtered viral particle supernatant at **50000 x g, 4°C, 01:30:00** . 1h 30m

11 Carefully transfer tubes on ice, mark pellet position/expected position of pellet and decant slowly to discard supernatant.

12 Resuspend pellet as a ~200X concentrate of total supernatant (e.g. 180 µL from 36 mL of supernatant).

As lentivirus particles are enveloped and more susceptible to degradation in comparison to unenveloped DNA viruses, the addition of 10% DMSO as a cryoprotectant allows at least 4X freeze-thaw cycles without compromising viral particles and reduces necessity for low volume aliquots (Figure 3).

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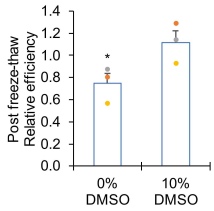

Figure 3. Inclusion of DMSO retains transfection efficiency of viral particle aliquots following 4X freeze-thaw cycles. Experiment performed with a hNGN2-eGFP containing plasmid (n = 3, error presented as SEM; * p < 0.05, comparison with Student's t-test).

13 Prepare viral particle aliquots of appropriate volumes corresponding to application and freeze at **-80 °C** .

Viral titre (TU/mL) can be calculated (# transfected cells / volume of virus) based on fluorescent reporter or immunofluorescent staining.

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