# Development and Optimization of a Novel Transfection Formulation for High Titer Recombinant Lentivirus and Adeno-associated Virus (AAV) Production

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## Abstract

The tremendous success in clinical trials and FDA approval of several gene therapies in 2017 has led to an increased interest in the optimization and scale-up of virus manufacturing processes to deliver large quantities of high titer recombinant lentivirus and AAV. To address this need, we screened our lipid and polymer libraries using a functional titer read-out to identify a novel transfection formulation that provides robust titers for lentivirus and AAV production in both adherent, serum-containing cultures, as well as serum-free suspension 293-derived cell types.

Scale-up and reproducibility are key attributes in large-scale manufacturing. Transfection complex formation is a critical step for transient transfection; key parameters including buffer composition, incubation time and the volume of the complex formation were assessed and optimized. Proof-of-principle scaling experiments were performed in suspension 293 cells from multi-well formats to large shake flasks. Reproducibility of the transfection process was also addressed through functional titer determination of multiple virus batches manufactured over an extended time period.

Different transfection technologies have different compositions, transfection efficiencies, virus production capabilities and subsequent functional titers. Multiple transfection technologies were compared in head-to-head studies for recombinant lentivirus and AAV production in suspension 293 cell types using functional titers. Virus genomes were also assessed to increase our understanding of the total population of virus that was produced using different transfection methods.

Our data demonstrate that transient transfection is a robust and reliable tool that can be harnessed for large-scale manufacturing of both recombinant lentivirus and AAV.

## Development Goals

**1** Best-in-class generation of recombinant AAV and lentivirus

**2** Compatible with suspension and adherent 293-derived cell lines

**3** Robust, scalable protocol

AAV2-GFP
(pAAV-hrGFP, pAAV-RC, and pAAV-Helper, Agilent)

AAV2 harvested exclusively from the cell lysate

3rd Generation Lentivirus
(MISSION® pLKO.1-puro-CMV-TurboGFP™ (Sigma), and pRSV-Rev, pCMV-VSV-G, pCgpV (Cell Bio Labs))

Lentivirus harvested exclusively from cell supernatant

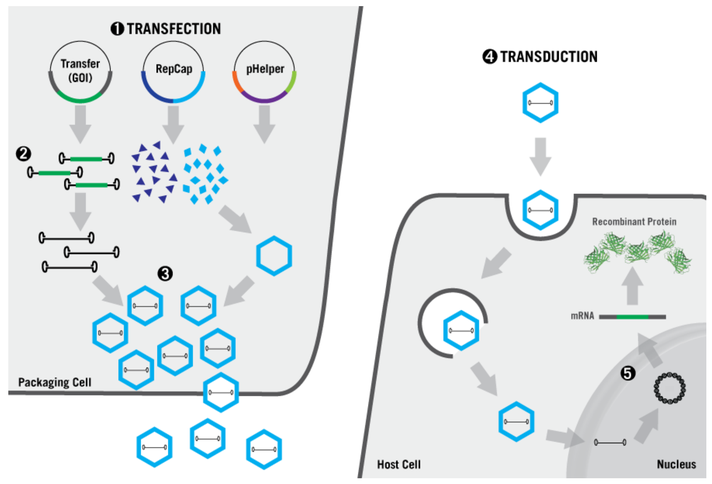
FreeStyle™ 293-F cells grown in FreeStyle™ F17 medium

ATCC HEK 293T/17 cells grown in DMEM + 10% FBS

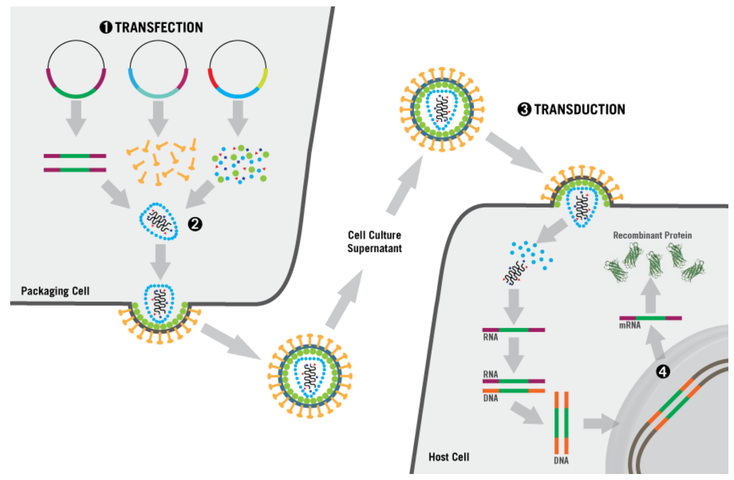
## Assays for Virus Detection

| | UNIT | AAV | Lentivirus |
|---|---|---|---|
| Functional: (infectious virus) | Transducing unit/ml (TU/ml) | | |
| qPCR: (virus genome) | Viral genomes/ml (vg/ml) | | |
| ELISA: (capsid) | Particles/ml (p/ml) | | |
| Transfection Efficiency: (plasmid DNA) | % GFP positive | Transfer (GOI) | Transfer (GOI) |

## Overview of Recombinant Adeno-associated Virus (AAV) and Lentivirus Particle Generation and Transduction

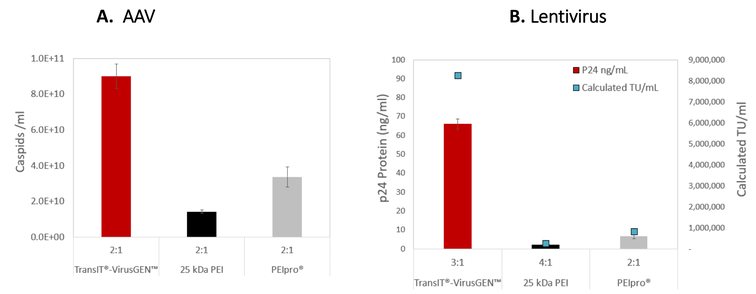



**Recombinant AAV Production Overview.** (1) Packaging cells (e.g.293T) are transfected with 3 plasmids encoding the gene of interest flanked by internal terminal repeats (ITRs), essential virus proteins (e.g. rep and cap), and a helper plasmid containing the adenovirus components. The ssDNA genome is replicated (2) and virus is assembled (3). A portion of the virus is released into the supernatant while some is retained within the cell. AAV can be purified from both the cell pellet and supernatant depending on the cell type. Typically AAV generated in suspension cells is isolated from only the cell pellet. (4) Permissive target cells, based on the AAV serotype, are transduced with recombinant AAV which enters through traditional endocytosis pathways. The virion then traffics to the nucleus where the ssDNA is released from the capsid (5). Transcription and translation result in the production of the protein encoded by the gene of interest.




**Recombinant Lentivirus Production Overview.** (1) Packaging cells (e.g.293T) are transfected with 3-4 plasmids encoding the gene of interest, vesicular stomatitis G protein (VSV-G) and essential virus proteins (e.g. gag, pol and rev). (2) Virus is assembled and released into the supernatant through budding with the producer cell plasma membrane resulting in an envelope decorated with VSV-G. The medium containing virus is filtered through a 0.45 µm filter to remove any cells. (3) Target cells are frequently transduced with recombinant lentivirus particles in the presence of a polycation to enhance efficiency. The virus enters the cell and the capsid is uncoated revealing the RNA genome and viral enzymes. The viral RNA is reverse transcribed into DNA which is then integrated into the host genome. (4) Transcription and translation result in the production of the protein encoded by the gene of interest.

## Capsid Quantification

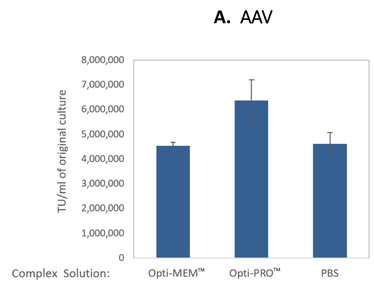


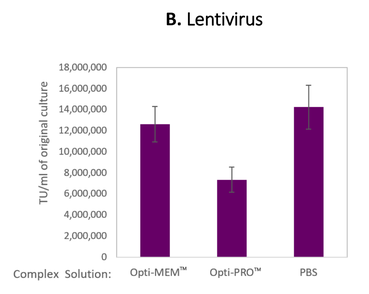




(A) For AAV2 production, suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium were transfected with pAAV-hrGFP, pAAV-RC, and pAAV-Helper (1:1:1 ratio, 1.5 µg/ml, Agilent Technologies) with *Trans*IT-VirusGEN® (2:1 vol:wt, Mirus Bio), 25 Kda Linear PEI (2:1 wt:wt, Polysciences) and PEIpro ® (2:1, vol:wt, Polyplus) using Opti-MEM™ for complex formation. AAV2 was harvested from the cell lysate (72 hpt) and capsids were quantitated using the AAV2 Titration ELISA (ProGen). (B) For lentivirus production, suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium were transfected with pLKO.1-puro-CMV-TurboGFP™ transfer vector and third generation packaging plasmids (total 1 µg/ml DNA) with *Trans*IT-VirusGEN® (3:1, vol:wt) 25 Kda Linear PEI (4:1 wt:wt, Polysciences) and PEIpro ® (2:1, vol:wt, Polyplus) using Opti-MEM for complex formation. The supernatant containing lentivirus was harvested (48hpt) and p24 levels were quantitated using the HIV Type 1 p24 Antigen ELISA (Zeptometrix). All conditions were performed in triplicate in non-treated 6-well plates (2 mL/well) shaking.

## Transfection Complex Formation Buffer

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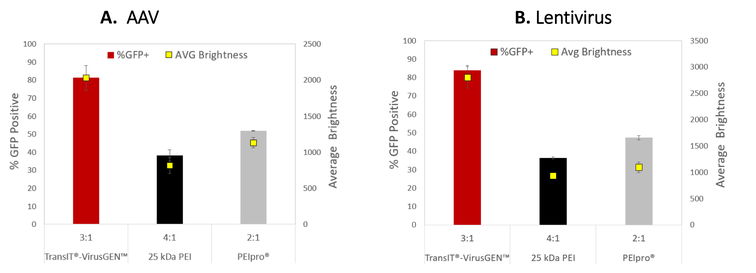
## Transfection Efficiency During Virus Production







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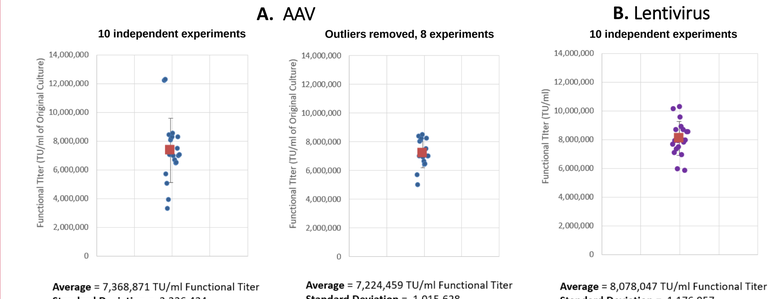
## Experimental Reproducibility

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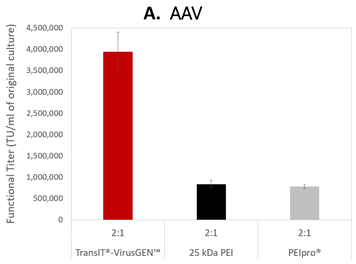
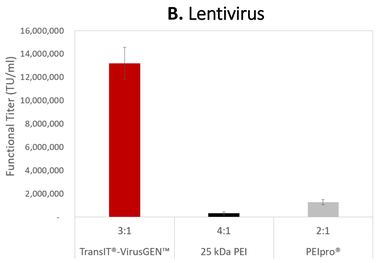
## Functional Virus Titer

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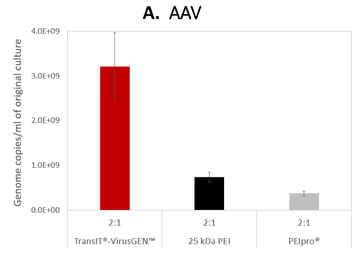
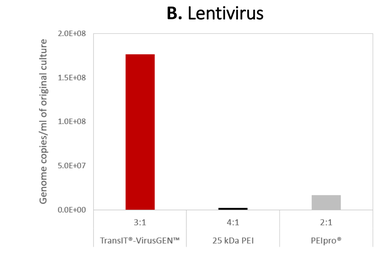
## Virus Genome Number

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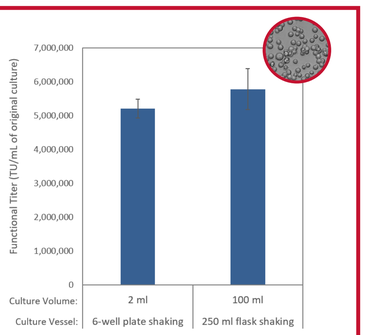
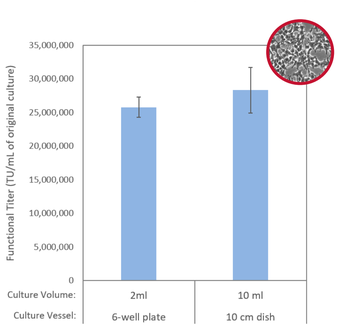
## Initial Scale-up Studies

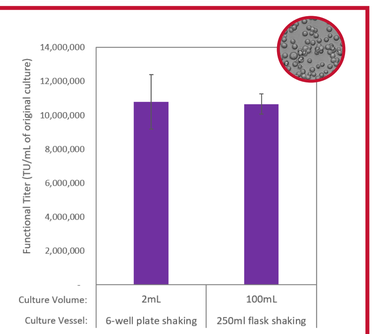
### AAV

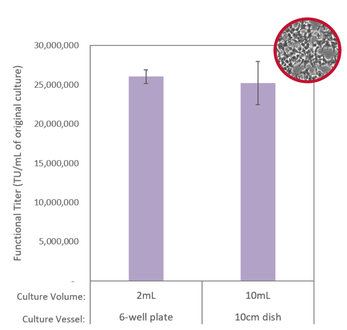



Suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium (A) or adherent 293T/17 cells (B) were transfected with pAAV-hrGFP, pAAV-RC, and pAAV-Helper (1:1:1 ratio, 1.5 total µg/ml, Agilent Technologies) using the TransIT®-VirusGEN™ (2:1 reagent-to-DNA ratio (vol:wt), Mirus) using the indicated volumes and culture vessel. Harvested virus was used to transduce HT1080 cells and GFP expression was measured 48 hours by flow cytometry. Functional titers were measured from virus dilutions with less than 20% GFP positive cells. The error bars represent the standard deviation of triplicate wells or dishes.

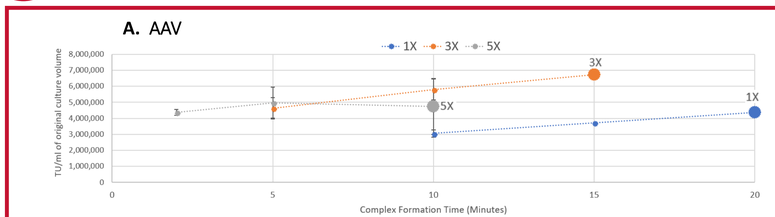
### Lentivirus

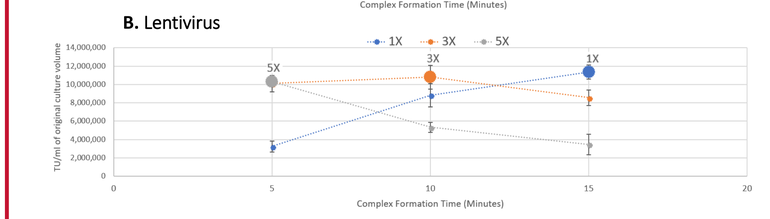



Suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium (A) or adherent 293T/17 cells (B) were transfected with pLKO.1-puro-CMV-TurboGFP™ transfer vector and the ViraSafe™ Lentiviral Packaging Systems (1:1 ratio, 1 µg/ml total) with TransIT®-VirusGEN™ (3:1 reagent-to-DNA ratio (vol:wt)) using the indicated volumes and culture vessel. 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 TransduceIT™ and GFP expression was measured 72 hours post-transduction using 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.

## Concentrated Transfection Complexes

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## Conclusions

- **High Functional Titers-** AAV and lentivirus produced in suspension and adherent 293-derived cells
- **Scalable –** Efficient across different formats
- **Reliable-** Consistent high titer production across independent experiments
- **Robust-** Compatible with multiple complex formation buffers and concentrations