Large-Scale Plasmid DNA Complexation Strategies for Commercial Lentiviral Production via Transient Transfection

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Abstract

Lentiviral (LV) vectors are a cornerstone of gene and cell therapy manufacturing, yet scalable and robust production remains a key challenge. In transient transfection workflows, plasmid DNA complexation is a critical step, where large-volume handling, mixing, and timing constraints can introduce variability and operational risk. We previously demonstrated successful scale-up of the CTS™ Gibco™ LV-MAX™ Lentiviral Production System to 50 L bioreactors. Here, we further streamline and de-risk transfection by evaluating reagent stability, complexation kinetics, and workflow design. To simplify processing, we developed a single-vessel workflow in which transfection reagent is diluted, mixed, and combined with neat DNA in a 10 L bag. Mixing was optimized using dye-based mimics, and resulting complexes showed comparable size, stability, and infectivity to the standard two-vessel method. Complexes also remained functional beyond the standard 10-minute preparation window, supporting an extended operational hold time. DNA–Transfection reagent (TFX-R) complexation was robust across temperatures, with particle size increasing at higher temperatures but no impact on kinetics or LV titers. LV-MAX system™ reagents remained stable at room temperature for at least 24 hours, enabling flexible staging without performance loss. Together, these results define a more flexible and simplified transfection strategy, enabling robust, scalable LV manufacturing with the LV-MAX™ system.

Materials and Methods

- LV-MAX™ Lentiviral Production System
- LV-MAX™ Lentiviral Packaging Mix
- Opti-MEM™
- ViralSEQ™ Lentivirus Physical Titer Kits
- 10 L Labtainer™ BioProcess Container (BPC), CX5-14 film

Lentiviral production
Lentiviral vectors were produced using the LV-MAX™ Lentiviral Production System according to the large scale protocol in the manual. Briefly, total of 2.5 ug plasmid DNA was used to transfect 4.0E+6 cells per mL of culture. The plasmid DNA consisted of a 1.5:1 ratio of LV-MAX™ lentiviral packaging mix and a GFP transfer plasmid. Transfections were performed in either 96-deep well plate or 125 mL flask formats (1mL and 30 mL volumes respectively). Crude lentiviral vectors were harvested 48 hours post-transfection by centrifugation at 13,000g for 15 min and stored at -80°C.

Transfection Efficiency
24 hours post-transfection, the fraction of GFP positive Viral Production Cells and mean fluorescent intensity of the live cell population were evaluated using the Attune™ NxT Flow Cytometer to determine transfection efficiency.

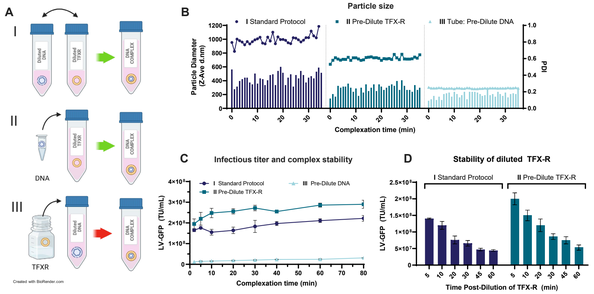
Infectious Lentiviral infective titration
Functional titer (TU/mL) was determined through an infectivity assay using Viral Production Cells. Crude lentiviral samples, harvested per the user guide, were thawed on ice, serially diluted in LV-MAX™ Production Medium and 0.1 mL of inoculum was used to transduce 27,000 cells/well in a flat-bottom 96-well microplate. Plates were incubated at 37°C/5% CO_2 for 48-72 hours before determining % GFP positive cells on the Attune™ NxT Flow Cytometer. Titer was calculated from samples falling in the 5-20% positive range.

Physical Lentiviral titration by RT-qPCR.
Quantitation of genome-containing lentiviral particles (VG/mL) was performed using a one-step RT-qPCR ViralSEQ™ Lentivirus Physical Titer Kit. The assay targets a conserved region (LTR) in the lentiviral vector genome. Crude harvested lentiviral vectors were prepared for the RT-qPCR using the PrepSEQ Nucleic Acid Sample Preparation Kit according to the published user guide.

Particle Size
Malvern Panalytical Zetasizer Nano ZS was used to measure particle size of the transfection complex by Dynamic Light Scattering (DLS) at a 173° Backscatter measurement angle, according to the user manual. Both particle size (Z-Ave d.nm) and polydispersity (PDI) were recorded over time and temperature was controlled were indicated. Fisherbrand™ Disposable Cuvettes (4.5 mL, standard cuvette, PMMA) were used to hold 1 mL of transfection complex for measurement.

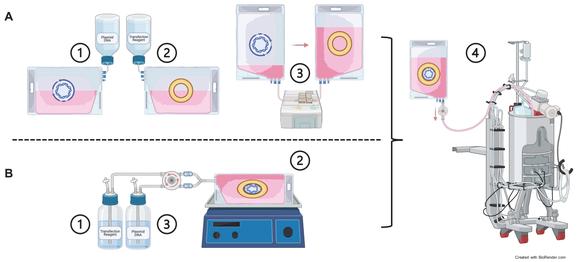
Results

1. DNA–Transfection reagent complexation strategy and impacts on particle size, complex stability, and lentiviral titer



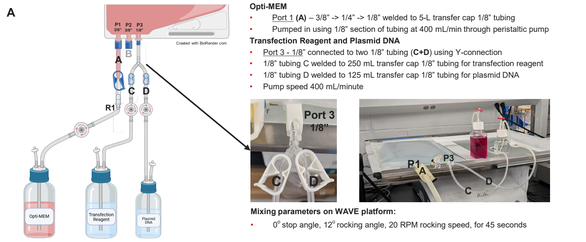
A) DNA–TFX-R complexation was performed using three setups: (I) standard 2-tube: DNA and TFX-R separately prediluted in Opti-MEM and then combined in either direction, (II) 1-tube protocol with diluted TFX-R plus undiluted DNA, or (III) 1-tube protocol with prediluted DNA plus undiluted TFX-R. **B)** Complex particle formation was monitored by DLS for 35 min. Size remained stable but differed by method: ~1000 nm (2-tube), ~700 nm (prediluted TFX-R), and ~300 nm (prediluted DNA), with corresponding PDI values. **C)** Infectious LV-GFP titers were highest with the prediluted TFX-R 1-tube protocol, exceeded the 2-tube method, and were markedly reduced with prediluted DNA. Complexes remained stable for >60 min post-DNA addition. **D)** Diluted TFX-R is unstable in Opti-MEM; LV-GFP titers decrease with increasing pre-complexation dilution times for both 1 and 2-tube protocols. Hence, DNA should be added after ~1 min equilibration but preferably no later than 5 min of TFX-R dilution.

2. Proposed workflow to improve process efficiency over the standard two-bag protocol



A) Current two-bag protocol (50L scale): (**1**) 100 mL DNA is diluted in 2 L Opti-MEM (bag 1) and (**2**) 240 mL TFX-R in 2 L Opti-MEM (bag 2). Bag 2 is sterile-welded and gravity-drained into bag 1 within 5 min (**3**), followed by manual mixing, 10 min incubation, and transfer to the bioreactor (**4**). **B)** Proposed single-bag process (50 L scale): 4 L Opti-MEM is preloaded into a 10 L bag, and (**1**) 240 mL TFX-R is added via peristaltic pump (400 mL/min). After 45 s wave mixing (12° angle, 20 rpm) (**2**) and 2 min incubation, (**3**) 100 mL undiluted DNA is added, mixed again (**2**), allowed to incubate for 10 min and transferred to the bioreactor (**4**).

3. Bag mixing study setup using dye mimetics



Opti-MEM
- Port 1 **(A)** – 3/8" -> 1/4" -> 1/8" welded to 5-L transfer cap 1/8" tubing
- Pumped in using 1/8" section of tubing at 400 mL/min through peristaltic pump

Transfection Reagent and Plasmid DNA
- Port 3 - 1/8" connected to two 1/8" tubing (**C**+**D**) using Y-connection
- 1/8" tubing C welded to 250 mL transfer cap 1/8" tubing for transfection reagent
- 1/8" tubing D welded to 125 mL transfer cap 1/8" tubing for plasmid DNA
- Pump speed 400 mL/minute

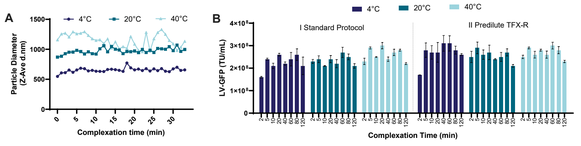
Mixing parameters on WAVE platform:
- 0° stop angle, 12° rocking angle, 20 RPM rocking speed, for 45 seconds

Optimal mixing in bioprocessing containers (BPC) is crucial for achieving high titers at bioreactor scale. Here, we utilized colorimetric changes to identify the best mixing strategy to achieve homogenous mixing of mock transfection components. Water was used as Opti-MEM, phenol red served as a proxy for transfection reagent, and 1 M NaOH was used as a DNA mimic. A setup of the connections is shown in **3A.** A 10 L BPC was connected to a 5 L Thompson flask containing 4 L water via port 1. The water was pumped into the bag which was then connected via port 3 to 2 bottles containing mock TFX-R and mock DNA using a Y-connector.

Step 1: Add 4 L Opti-MEM to 10 L Labtainer BPC
Step 2: Mock TFX-R addition 240 mL
TFX-R addition complete after 45 seconds
Step 3: Agitation by rocking
Step 4: Mock plasmid DNA addition 100 mL
DNA addition complete after 20 seconds
Step 5: Agitation by rocking, begin incubation

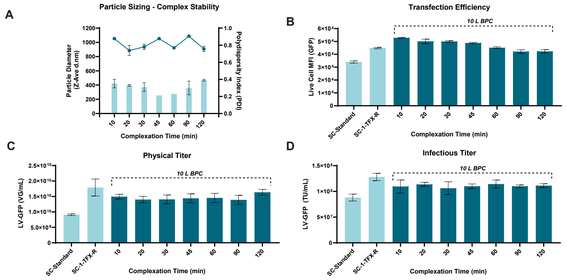
B) As a first step, 240 mL of mock TFX-R was pumped into 4L water at 400 mL/min. Transfer was complete after 45 seconds and the flow rate of 400 mL/minute resulted in proper dispersion of the mock TFX-R. A lower pump speed had suboptimal dispersion (not shown). Complete mixing was observed after 45 seconds of rocking at 20 rpm and 12°. The mixture was allowed to sit for 3 minutes after rocking during which the pump line was switched to the mock DNA solution. Mock DNA was pumped in at the same 400 mL/min flow rate and transferred in 20 seconds, after which another round of rocking was performed to complete the mixing. This timepoint was considered the start of the complexation incubation.

4. Temperature affects particle size but not complexation kinetics or lentiviral yield.



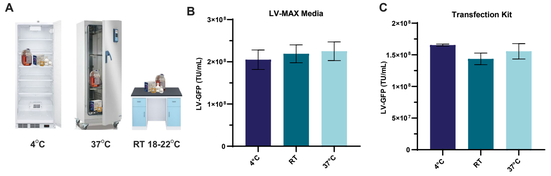
A) Particle formation was assessed by DLS after pre-equilibrating reagents and performing complexation at indicated temperatures. Particle size increased with temperature (~600 nm at 4°C to ~1200 nm at 37°C), while complexation kinetics and stability were unaffected over 35 min. **B)** Functional impact was evaluated by generating LV-GFP following complexation at 4°C, 20°C, or 40°C. Complexes remained stable across temperatures without effects on viral titer. Thus, complexation is temperature-tolerant, can be performed at room temperature without compromising stability or yield. The standard 10 min complexation time can be extended to at least 1 hr, but should not be less than 5 minutes.

5. Large complexation protocol results in stable complex formation and high titer LV-production.



Complexation was performed in a 10 L BPC using the single-vessel workflow, representing a process used for 50 L production scale transfections. At defined timepoints (10–120 min post-complexation), aliquots were withdrawn directly from the BPC via peristaltic pump (~200 mL/min) and used to transfect Viral Production Cells in 125 mL flasks (n=3). In parallel, small-scale control complexes were prepared using the standard (SC-Standard (Fig 1A I)) and single-vessel, predilute TFX-R protocol (SC-1-TFX-R (Fig 1A II)) and allowed to complex for 10 min prior to transfection. LV-GFP was harvested 48 h post-transfection. **A)** Complex size was assessed by DLS. Particle size (~900 nm) remained stable for up to 2 hr. post-complexation. **B)** Transfection efficiency measured by GFP expression at 24 hr post-transfection. The single-vessel (10 L BPC) protocol performed comparably to the standard workflow small scale controls across all timepoints. **C)** Physical titer (VG/mL) quantified by RT-qPCR. Genome titers were stable across timepoints, with a modest increase observed for the single-vessel condition relative to standard. **D)** Functional titer determined by infectivity assay. Infectious titers of the single-vessel workflow were comparable to or slightly higher than the standard protocol across all timepoints.

6. LV-Max™ system component temperature stability allows for prestaging of components



A) LV-MAX™ Production Medium (1 L bottles) and LV-MAX™ Transfection Kits (1 L kits) including Opti-MEM were stored at 4°C, room temperature (18–22°C), or 37°C protected from light prior to use. Following storage at the designated condition, reagents were used in a standard transfection protocol at the 125 mL scale. No significant impact on infective titer was observed following **B)** 7 d exposure of media or **C)** 24 h exposure of transfection components.

Conclusion

This study establishes a simplified and robust DNA–transfection reagent complexation workflow for large-scale lentiviral production using the Gibco™ CTS™ LV-MAX™ system, demonstrating tolerance to temperature, extended hold times, and room-temperature reagent stability. The single-vessel approach slightly increases LV-GFP viral titers while reducing process complexity and operational risk.

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