High Titer Recombinant Lentivirus and Adeno-associated Virus Production for Therapeutic Applications

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Abstract

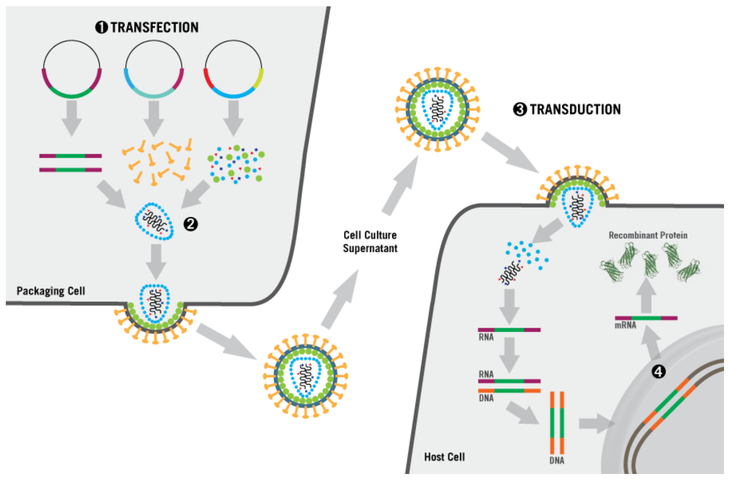
Transient transfection is a robust and reliable tool for the manufacture of recombinant lentivirus and adeno-associated virus (AAV). The therapeutic promise of these technologies through the modification of relevant immune cells or overexpression of a target gene drives the demand for enhanced virus production methodologies. Recognizing this need, Mirus Bio has developed a novel transfection formulation, *Trans*IT-VirusGEN®, specifically for high titer manufacture of recombinant lentivirus and AAV in 293-derived cell types. In addition, movement of recombinant virus production into the gene and cell therapy manufacturing arena increases the focus on quality parameters such as reproducibility and raw material testing. To address some of these criteria, lot-to-lot consistency, identity and safety testing of the transfection formulation were assessed.

To further support high yield recombinant lentivirus and AAV production, we identified novel enhancers and a complex formation solution that increase functional virus titers 2-3 fold over previously optimized high titer conditions. These parameters were tested with multiple serum-free media formulations and examined using different plasmid DNA concentrations to better understand the compatibility within a workflow. In addition, head-to-head comparisons of this novel system were performed with commonly used transfection technologies to accurately assess the recombinant virus production capabilities.

Our data demonstrate that we can push the limits of high titer recombinant lentivirus and AAV production to increase manufacturing capabilities for gene and cell therapy applications.

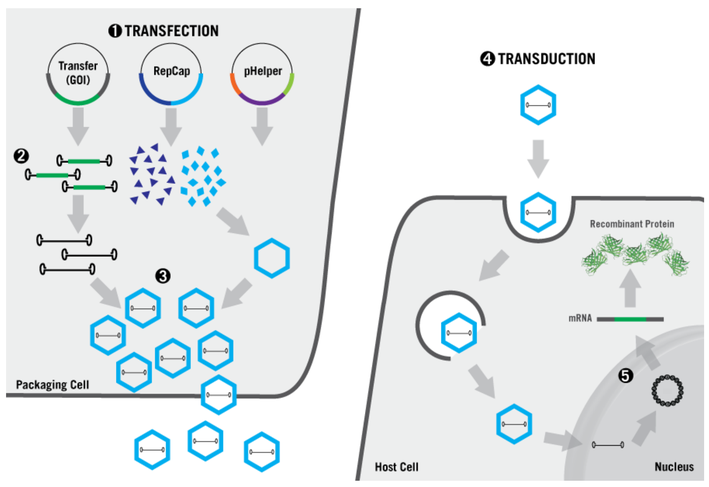
Recombinant Virus Production Overview

Lentivirus



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.

AAV



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.

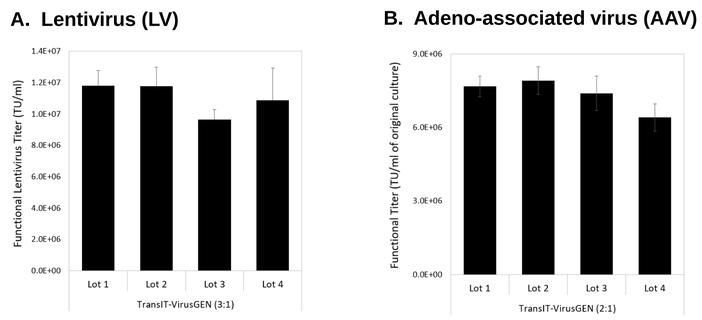
*Trans*IT-VirusGEN® Transfection Reagent

The following chart illustrates how all configurations of *Trans*IT-VirusGEN® Transfection Reagent can help support the biopharma market through each stage of the biotherapeutic pipeline from process development through late stage clinical trial and manufacturing. Each stage is defined by an appropriate configuration.

Description	*Trans*IT-VirusGEN®	*Trans*IT-VirusGEN® SELECT	*Trans*IT-VirusGEN® GMP
Biotherapeutic Pipeline Support	Process development & pre-clinical	Pre-clinical & early phase clinical trial	Late-phase clinical trial & commercial manufacturing
Composition	Ready to use, chemically defined and animal origin free		
Quality Grade	R&D grade	Pre-clinical grade	GMP grade
Packaging	Vials	Bottles	TBD
Configuration	0.3mL, 0.75mL, 1.5mL 5x and 10x1.5mL	30mL (custom options available)	TBD
Quality Control	Standard functional QC	Includes additional quality testing such as sterility according to USP <71>, bacterial endotoxin, according to USP <85>, mycoplasma according to USP <63> and product identity.	Manufactured as a quality-assured product according to relevant guidelines for Good Manufacturing Practice (GMP).
Availability	NOW	Summer 2019	2020

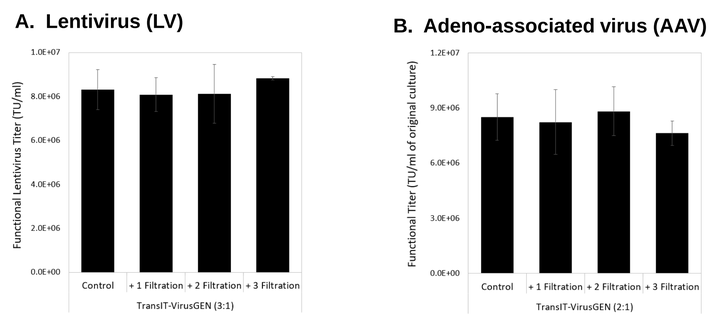
Same formulation – sample *Trans*IT-VirusGEN® now

Robust Lot-to-Lot Consistency

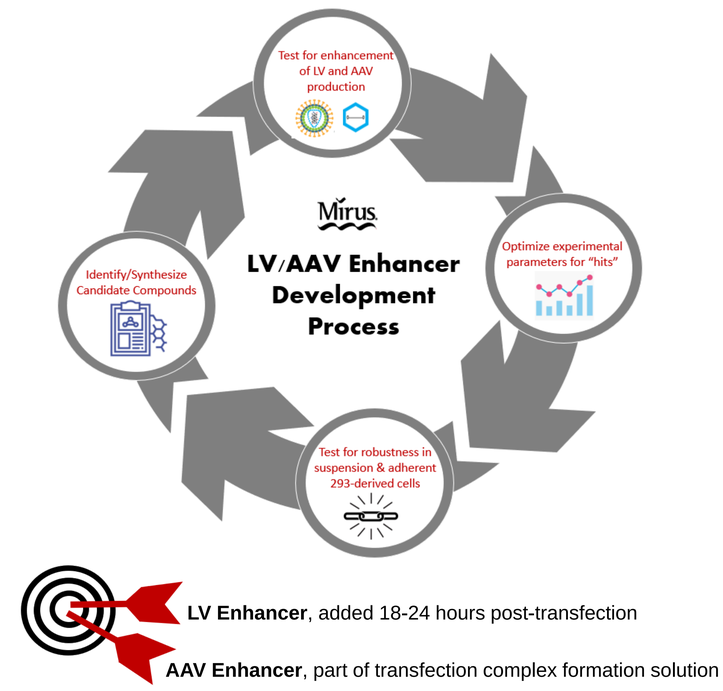


Reliable Lot-to-Lot Consistency with the *Trans*IT-VirusGEN® Transfection Reagent. (A) Lentivirus was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium and transfected with 3rd generation vectors pLKO.1-puro-CMV-TurboGFP™ transfer vector (Sigma) and ViraSafe Pantropic Packaging mix (pRSV-Rev, pCMV-VSV-G, pCgpV, Cell Bio Labs) at a 3:0.5:0.5:2 DNA ratio, 1 µg/ml total plasmid, using the *Trans*IT-VirusGEN® Transfection reagent (3:1, vol:wt). Virus containing supernatant was used to transduce 293T/17 cells and GFP expression was measured at 72 hours post post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. (B) AAV2 was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium and transfected using pAAV-hrGFP, pAAV-RC, and pAAV-Helper plasmids (1:1:1 DNA ratio, 1.5 µg/ml, Agilent Technologies) using *Trans*IT-VirusGEN® (2:1, vol:wt). Harvested virus was used to transduce HT1080 cells and GFP expression was measured 48 hours post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. For both lentivirus and AAV functional titers were measured from virus dilutions with less than 20% GFP positive cells. The error bars represent the standard deviation of triplicate wells.

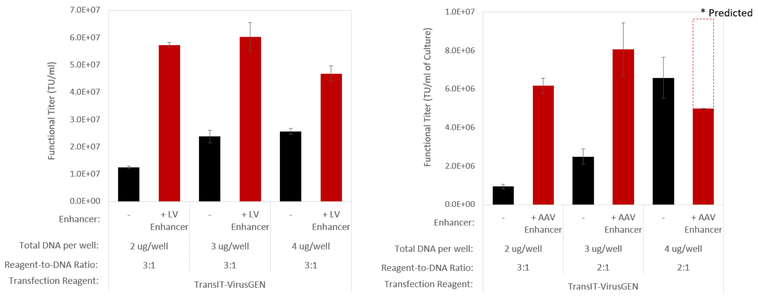
No Effect of Multiple Filtrations



***Trans*IT-VirusGEN® Transfection Reagent can be Filtered Multiple Times.** (A) Lentivirus was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium and transfected with 3rd generation vectors pLKO.1-puro-CMV-TurboGFP™ transfer vector (Sigma) and ViraSafe Pantropic Packaging mix (pRSV-Rev, pCMV-VSV-G, pCgpV, Cell Bio Labs) at a 3:0.5:0.5:2 DNA ratio, 1 ug/ml total plasmid, using the *Trans*IT-VirusGEN® Transfection reagent (3:1, vol:wt) that was filtered through a 0.22 um polyethersulfone (PES) filter unit (Millipore Sigma) for the indicated number of times. Virus containing supernatant was used to transduce 293T/17 cells and GFP expression was measured at 72 hours post post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. (B) AAV2 was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium and transfected using pAAV-hrGFP, pAAV-RC, and pAAV-Helper plasmids (1:1:1 DNA ratio, 1.5 µg/ml, Agilent Technologies) using *Trans*IT-VirusGEN® (2:1, vol:wt). Harvested virus was used to transduce HT1080 cells and GFP expression was measured 48 hours post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. For both lentivirus and AAV functional titers were measured from virus dilutions with less than 20% GFP positive cells. The error bars represent the standard deviation of triplicate wells.

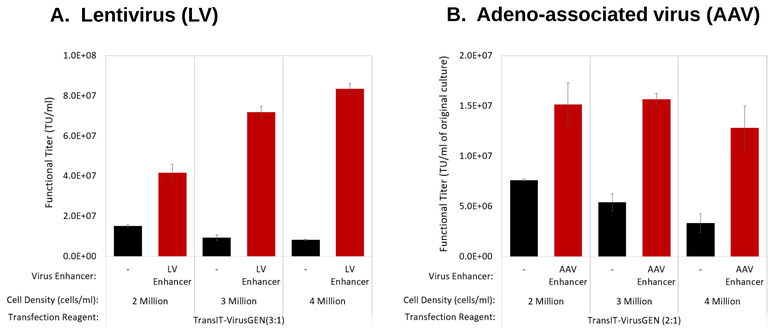


Effect of DNA Concentration



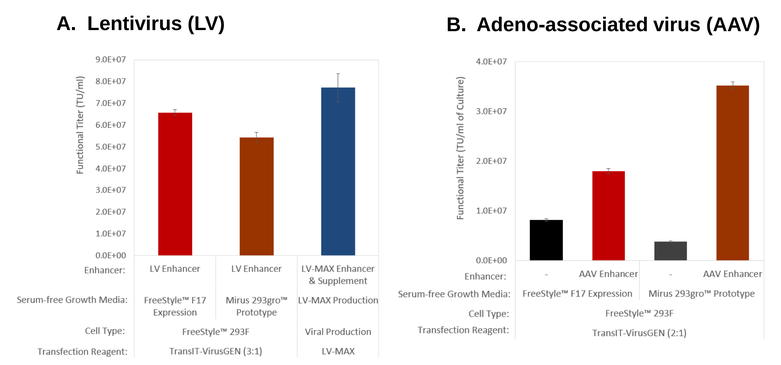
Plasmid DNA Titration using the TransIT-VirusGEN® Transfection Reagent +/- LV or AAV Enhancer. (A) Lentivirus was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium and transfected with 3rd generation vectors pLKO.1-puro-CMV-TurboGFP™ transfer vector (Sigma) and ViraSafe Pantropic Packaging mix (pRSV-Rev, pCMV-VSV-G, pCgpV, CellBio Labs) at a 3:0.5:0.5:2 DNA ratio using the *Trans*IT-VirusGEN® Transfection reagent (3:1, vol:wt). The amount of DNA was titrated from 2 µg/well (2 ml at 1 µg/ml), 3 µg/well or 4 µg/well while maintaining the reagent-to-DNA ratio of 3:1. Cells were transfected at a cell density of 2 million cells/ml. The LV Enhancer was added 20 hours post-transfection. Virus containing supernatant was used to transduce 293T/17 cells and GFP expression was measured at 72 hours post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. (B) AAV2 was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium and transfected using pAAV-hrGFP, pAAV-RC, and pAAV-Helper plasmids (1:1:1 DNA ratio, Agilent Technologies) using *Trans*IT-VirusGEN® (2:1, vol:wt). The amount of DNA was titrated from 2 µg/well (2 ml at 1 µg/ml), 3 µg/well or 4 µg/well and the optimal reagent-to-DNA ratio of 3:1 or 2:1 is shown for each condition. Cells were transfected at a cell density of 2 million cells/ml and the AAV enhancer is part of the complex formation solution. Harvested virus was used to transduce HT1080 cells and GFP expression was measured 48 hours post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. The exception is noted by a an asterisk (*) and is a predicted value since too low of a dilution was performed for this sample. For both lentivirus and AAV functional titers were measured from virus dilutions with less than 20% GFP positive cells. The error bars represent the range of duplicate wells.

Cell Density at the Time of Transfection



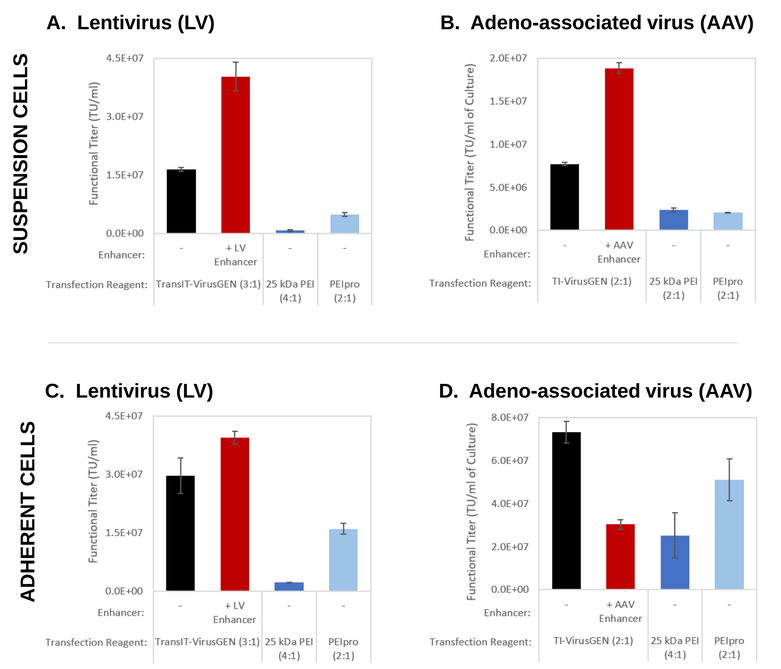
Cell Density Titration at the Time of Transfection using the *Trans*IT-VirusGEN® Transfection Reagent +/- LV or AAV Enhancers. (A) Lentivirus was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium and transfected with 3rd generation vectors pLKO.1-puro-CMV-TurboGFP™ transfer vector (Sigma) and ViraSafe Pantropic Packaging mix (pRSV-Rev, pCMV-VSV-G, pCgpV, Cell Bio Labs) at a 3:0.5:0.5:2 DNA ratio, 1 µg/ml = 2 µg/well, using the *Trans*IT-VirusGEN® Transfection reagent (3:1, vol:wt). The cell density at the time of transfection was titrated from 2 million to 4 million cells/ml. The LV Enhancer was added 20 hours post-transfection. Virus containing supernatant was used to transduce 293T/17 cells and GFP expression was measured at 72 hours post post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. (B) AAV2 was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium and transfected using pAAV-hrGFP, pAAV-RC, and pAAV-Helper plasmids (1:1:1 DNA ratio, 1.5 µg/ml = 3 µg/well, Agilent Technologies) using *Trans*IT-VirusGEN® (2:1, vol:wt). The cell density at the time of transfection was titrated from 2 million to 4 million cells/ml. Harvested virus was used to transduce HT1080 cells and GFP expression was measured 48 hours post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. For both lentivirus and AAV functional titers were measured from virus dilutions with less than 20% GFP positive cells. The error bars represent the range of duplicate wells.

Serum-free Complete Media Formulation



Serum Free Complete Media Comparison using the *Trans*IT-VirusGEN® Transfection Reagent LV Enhancer or +/- AAV Enhancers.. (A) Lentivirus was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium or Mirus 293gro™ Prototype Media and transfected with 3rd generation vectors pLKO.1-puro-CMV-TurboGFP™ transfer vector (Sigma) and ViraSafe Pantropic Packaging mix (pRSV-Rev, pCMV-VSV-G, pCgpV, Cell Bio Labs) at a 3:0.5:0.5:2 DNA ratio, 1 µg/ml = 2 µg/well, using the *Trans*IT-VirusGEN® Transfection reagent (3:1, vol:wt). The LV Enhancer was added 20 hours post-transfection. This was compared to the LV-MAX Production System using the same third generation vectors, 2.5 µg/ml = 5 µg/well using the LV-MAX Transfection Reagent (6:2.5, vol:wt). Viral Production cells grown in LV-MAX Production Media were transfected at a cell density of 4 million cells/ml containing 5% supplement. The LV-MAX Enhancer was added at 5 hours post-transfection. Virus containing supernatant was used to transduce 293T/17 cells and GFP expression was measured at 72 hours post post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. (B) AAV2 was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium or Mirus 293gro™ Prototype Media and transfected using pAAV-hrGFP, pAAV-RC, and pAAV-Helper plasmids (1:1:1 DNA ratio, 1.5 µg/ml = 3 µg/well, Agilent Technologies) using *Trans*IT-VirusGEN® (2:1, vol:wt). Cells were transfected at a cell density of 2 million cells/ml. Harvested virus was used to transduce HT1080 cells and GFP expression was measured 48 hours post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. For both lentivirus and AAV functional titers were measured from virus dilutions with less than 20% GFP positive cells. The error bars represent the range of duplicate wells.

Competitor Benchmarking



Competitor Comparison Experiments with *Trans*IT-VirusGEN® Reagent +/- LV or AAV Enhancers, 25 kDa linear PEI, and PEIpro® Transfection Reagents. (A) Lentivirus was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium or (C) adherent 293T/17 cells grown in DMEM + 10% FBS and transfected with 3rd generation vectors pLKO.1-puro-CMV-TurboGFP™ transfer vector (Sigma) and ViraSafe Pantropic Packaging mix (pRSV-Rev, pCMV-VSV-G, pCgpV, Cell Bio Labs) at a 3:0.5:0.5:2 DNA ratio, 1 µg/ml = 2 µg/well, using the *Trans*IT-VirusGEN® Transfection reagent (3:1, vol:wt). The LV Enhancer was added 20 hours post-transfection. Virus containing supernatant was used to transduce 293T/17 cells and GFP expression was measured at 72 hours post post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. (B) AAV2 was produced using suspension FreeStyle™ 293-F cells grown in FreeStyle™ F17 Medium (D) adherent 293T/17 cells grown in DMEM + 10% FBS and transfected using pAAV-hrGFP, pAAV-RC, and pAAV-Helper plasmids (1:1:1 DNA ratio, 1.5 µg/ml = 3 µg/well, Agilent Technologies) using *Trans*IT-VirusGEN® (2:1, vol:wt). The AAV Enhancer is part of the complex formation solution, no separate addition is necessary. Harvested virus was used to transduce HT1080 cells and GFP expression was measured 48 hours post-transduction using Guava© easyCyte™ 5HT Flow Cytometer. For both lentivirus and AAV functional titers were measured from virus dilutions with less than 20% GFP positive cells. The error bars represent the range of duplicate wells.

Conclusions

- **Higher Grade Raw Material-** *Trans*IT-VirusGEN® SELECT Transfection Reagent is the same formulation as the *Trans*IT-VirusGEN® Transfection Reagent and includes additional quality testing including sterility, endotoxin, mycoplasma and identity testing.
- **Robust Transfection Reagent-** Minimal lot-to-lot variability for LV and AAV production and can be filtered multiple times to accommodate various workflow.
- **Novel VirusGEN LV and AAV Titer Enhancers for High Titers in Suspension Cells-** Combine the novel LV and AAV Enhancers for even higher titers. The LV Enhancer is a single addition at 18-24 hours post-transfection and the AAV enhancer is part of the complex formation solution.
- **Mirus Prototype 293gro™ Media Supports Virus Production -** Mirus 293gro Prototype Media supports robust production of LV and AAV in suspension 293 cell culture.