*Trans*IT-VirusGEN® Transfection Reagent

Quick Reference Protocol for AAV Generation

Instructions for MIR 6700, 6703, 6704, 6705, 6706, 6710

Full protocol, SDS and Certificate of Analysis available at mirusbio.com/6700

SPECIFICATIONS

Storage	Store *Trans*IT-VirusGEN® Transfection Reagent tightly capped at –20°C. ***Before each use***, warm to room temperature and vortex gently.
Product Guarantee	6 months from the date of purchase, when properly stored and handled.

PROTOCOL FOR ADENO-ASSOCIATED VIRUS (AAV) GENERATION IN ADHERENT 293T CELL CULTURES

Full protocol and additional documentation available at ***mirusbio.com/6700***

Fill in volumes below based on culture vessel used for transfection (Table 1).

A. Plate cells approximately 18-24 hours prior to transfection

1. Plate cells in ___ml complete growth medium (per well or flask).
   For HEK 293T/17 cultures: Plate cells at a density of 4.0—5.0 x 10^5 cells/ml
2. Culture overnight. Cells should be 80-95% confluent on day of transfection. Transfecting cells at a lower confluency may lead to high cellular toxicity and lower virus titers.

B. Prepare *Trans*IT-VirusGEN®:DNA complexes (Immediately before transfection)

1. Warm *Trans*IT-VirusGEN® Transfection Reagent to room temperature and vortex gently.
2. Place ___µl serum-free medium (e.g. OptiMEM® I Reduced-Serum Medium) in a sterile tube.
3. In a separate tube, combine all AAV plasmids per the manufacturer recommendations to a final concentration of 1 µg/µl. Mix gently by pipetting.
4. Transfer ___µl AAV DNA mixture (from B.3) to the tube containing serum-free medium. Mix gently by pipetting.
5. Add ___µl *Trans*IT-VirusGEN® to the diluted DNA mixture. Mix gently by pipetting.
6. Incubate at room temperature for 10-15 minutes to allow transfection complexes to form.

C. Distribute complexes to cells

1. Add *Trans*IT-VirusGEN®:DNA complexes drop-wise to different areas of the well.
2. Gently rock plate or vessel for even distribution of complexes.
3. Incubate at 37°C in 5% CO_2 for 72 hours prior to AAV harvest. NOTE: It is not necessary to replace the complete growth medium with fresh medium post-transfection.

D. Harvest and storage of AAV

1. Following the 72-hour incubation, prepare a dry ice/ethanol bath.
2. Remove cells from the plate using a cell scraper. Transfer cells and media to a new sterile tube.
3. To ensure thorough lysis of cells, freeze cells completely in the dry ice/ethanol bath and then thaw in a 37°C water bath. Perform freeze/thaw cycle a total of three times.
4. Centrifuge the cell lysate at 10,000 x g for 10 minutes to remove cell debris. Carefully transfer the supernatant containing AAV to a new sterile tube.
5. Store AAV stocks at -80°C.

Table 1. Recommended starting conditions

Culture vessel	6-well plate	10-cm dish	T75 flask
Surface area	9.6 cm^2	59 cm^2	75 cm^2
Complete growth medium	2.0 ml	10 ml	15 ml
Serum-free medium	200 µl	1.0 ml	1.5 ml
AAV Plasmid DNA (Pre-mixed, 1 µg/µl stock)	3.0 µl	15 µl	22.5 µl
***Trans*IT-VirusGEN® Reagent**	6 µl	30 µl	45 µl

Transfection Optimization

Determine the best *Trans*IT-VirusGEN® Reagent:DNA ratio for each cell type. Start with 2 µl of *Trans*IT-VirusGEN® per 1 µg of DNA. Vary the concentration of *Trans*IT-VirusGEN® from 1.5 – 4 µl per 1 µg DNA to find the optimal ratio.

For additional transfection optimization tips, see the TransIT-VirusGEN® full protocol.

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Quick Reference Protocol for AAV Generation

Instructions for MIR 6700, 6703, 6704, 6705, 6706, 6710

Full protocol, SDS and Certificate of Analysis available at mirusbio.com/6700

PROTOCOL FOR ADENO-ASSOCIATED VIRUS (AAV) GENERATION IN SUSPENSION 293 CELL CULTURES

Full protocol and additional documentation available at ***mirusbio.com/6700***

Fill in volumes below based on total culture volume (Table 2).

A. Maintenance of cells

1. Passage suspension 293 cells 18-24 hours prior to transfection.
2. Incubate cells overnight at appropriate temperature and CO_2 levels (e.g. 37°C, 5-8% CO_2, shaking).

B. Prepare *Trans*IT-VirusGEN® Reagent:DNA complexes

1. Seed cells at a density of 2 x 10^6 cells/ml immediately prior to transfection.
2. Warm *Trans*IT-VirusGEN® Transfection Reagent to room temperature and vortex gently.
3. Place ___ml of serum-free medium (e.g. Opti-MEM® I Reduced-Serum Medium) in a sterile tube.
4. In a separate tube, combine all AAV plasmids per the manufacturer recommendations to a final concentration of 1 µg/µl. Mix gently by pipetting.
5. Transfer ___µl AAV plasmid DNA mixture to the tube containing serum-free medium. Mix gently by pipetting.
6. Add ___µl of *Trans*IT-VirusGEN® Reagent to the diluted DNA. Mix gently by pipetting.
7. Incubate at room temperature for 10-15 minutes to allow transfection complexes to form.

C. Distribute complexes to cells in complete growth medium

1. Add *Trans*IT-VirusGEN® Reagent:DNA complexes to cultured cells (prepared in Step B.1).
2. Shake cultures on an orbital shaker (e.g. 125 rpm when using a shaker with a 1.9 cm orbital throw) at appropriate temperature and CO_2 levels (e.g. 37°C, 5-8% CO_2, shaking).
3. Incubate transfected cultures for 72 hours prior to AAV harvest.

D. Virus harvest and storage

1. Prepare a dry ice/ethanol bath and cell lysis buffer (50 mM Tris pH 8.5, 150 mM NaCl).
2. Transfer the cell suspension to a sterile tube. Centrifuge at 1,750 x g for 10 minutes.
3. Remove and discard the supernatant (AAV is contained within the cells before lysis).
4. Determine the volume of cell lysis buffer required for pellet resuspension by multiplying the transfected cell culture volume by 0.2 ml. Add cell lysis buffer to the cell pellet and mix thoroughly. (Example: Add 5.5 ml cell lysis buffer to a cell pellet from a 27.5 ml transfected culture.)
5. To ensure sufficient lysis of cells, freeze cells completely in the dry ice/ethanol bath and then thaw in a 37°C water bath. Perform freeze/thaw cycle a total of three times.
6. Add 50 U/ml Benzonase® to the cell lysate. Mix thoroughly but gently.
7. Incubate at room temperature for 30 minutes.
8. Centrifuge the cell lysate at 10,000 x g for 10 minutes to remove cell debris.
9. Transfer the AAV containing supernatant to a new sterile tube. Store AAV stocks at –80°C.

Table 2. Volume scaling worksheet for AAV generation using *Trans*IT-VirusGEN® Reagent.

Starting conditions per milliliter of complete growth medium (AAV Generation)					
	Per 1 ml		Total culture volume		Reagent quantities
Serum-free Complex Medium	0.1 ml	×	_____ml	=	_____ml
Total Plasmid DNA (1 µg/µl stock)	1.5 µl	×	_____ml	=	_____µl
*Trans*IT-VirusGEN® Reagent	3 µl	×	_____ml	=	_____µl

NOTE: Total Plasmid DNA refers to the combined mass of packaging plasmids + GOI containing transfer plasmids (in µg) per transfection.

*Trans*IT-VirusGEN® Transfection Reagent

Quick Reference Protocol for Lentivirus Generation

Instructions for MIR 6700, 6703, 6704, 6705, 6706, 6710

Full protocol, SDS and Certificate of Analysis available at mirusbio.com/6700

SPECIFICATIONS

Storage	Store *Trans*IT-VirusGEN® Transfection Reagent tightly capped at –20°C. ***Before each use***, warm to room temperature and vortex gently.
Product Guarantee	6 months from the date of purchase, when properly stored and handled.

PROTOCOL FOR LENTIVIRUS GENERATION IN ADHERENT 293T CELL CULTURES

Full protocol and additional documentation available at ***mirusbio.com/6700***

Fill in volumes below based on culture vessel used for transfection (Table 3).

A. Plate cells approximately 18-24 hours prior to transfection

1. Plate cells in ___ml complete growth medium (per well or flask).
   For HEK 293T/17 cultures: Plate cells at a density of 4.0—5.0 x 10^5 cells/ml
2. Culture overnight. Cells should be 80-95% confluent on day of transfection. Transfecting cells at a lower confluency may lead to high cellular toxicity and lower virus titers.

B. Prepare *Trans*IT-VirusGEN®:DNA complexes (Immediately before transfection)

1. Warm *Trans*IT-VirusGEN® Transfection Reagent to room temperature and vortex gently.
2. Place ___µl serum-free medium (e.g. OptiMEM® I Reduced-Serum Medium) in a sterile tube.
3. In a separate tube, combine ___µl packaging plasmid premix and ___µl transfer plasmid DNA encoding the gene of interest (GOI). Mix gently by pipetting.
4. Transfer the total volume of packaging premix + transfer plasmid mixture to the tube containing serum-free medium. Mix gently by pipetting.
5. Add ___µl *Trans*IT-VirusGEN® to the diluted DNA mixture. Mix gently by pipetting.
6. Incubate at room temperature for 10-15 minutes to allow transfection complexes to form.

C. Distribute complexes to cells

1. Add *Trans*IT-VirusGEN®:DNA complexes drop-wise to different areas of the well.
2. Gently rock plate or vessel for even distribution of complexes.
3. Incubate 48 hours prior to lentivirus harvest. NOTE: It is not necessary to replace complete growth medium with fresh medium post-transfection.

D. Harvest and storage of lentivirus

1. Harvest cell supernatant containing recombinant lentivirus particles.
2. Filter virus-containing supernatant through a 0.45 µm PVDF filter to remove any cells.
3. Immediately flash freeze aliquots in cryogenic tubes and store at –80°C.

Table 3. Recommended starting conditions

Culture vessel	6-well plate	10-cm dish	T75 flask
Surface area	9.6 cm^2	59 cm^2	75 cm^2
Complete growth medium	2.0 ml	10 ml	15 ml
Serum-free medium	200 µl	1.0 ml	1.5 ml
Transfer DNA (1 µg/µl stock)	1.0 µl	5.0 µl	7.5 µl
Packaging DNA Premix (1 µg/µl stock)	1.0 µl	5.0 µl	7.5 µl
***Trans*IT-VirusGEN® Reagent**	6 µl	30 µl	45 µl

Transfection Optimization

The amount of *Trans*IT-VirusGEN® required for transfection is dictated by the amount of DNA. Determine the best *Trans*IT-VirusGEN® Reagent:DNA ratio for each cell type. Start with 3 µl of *Trans*IT-VirusGEN® per 1 µg of DNA. Vary the concentration of *Trans*IT-VirusGEN® from 2–4 µl per 1 µg DNA to find the optimal ratio.

For additional transfection optimization tips, see the TransIT-VirusGEN® full protocol.

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Quick Reference Protocol for Lentivirus Generation

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Full protocol, SDS and Certificate of Analysis available at mirusbio.com/6700

PROTOCOL FOR LENTIVIRUS GENERATION IN SUSPENSION 293 CELL CULTURES

Full protocol and additional documentation available at ***mirusbio.com/6700***

Fill in volumes below based on total culture volume (Table 4).

A. Maintenance of cells

1. Passage suspension 293 cells 18-24 hours prior to transfection to ensure that cells are actively dividing at the time of transfection.
2. Incubate cells overnight at appropriate temperature and CO_2 levels (e.g. 37°C, 5-8% CO_2, shaking).

B. Prepare *Trans*IT-VirusGEN® Reagent:DNA complexes

1. Seed cells at a density of 2 x 10^6 cells/ml immediately prior to transfection.
2. Warm *Trans*IT-VirusGEN® Transfection Reagent to room temperature and vortex gently.
3. Place ___ml of serum-free medium (e.g. Opti-MEM® I Reduced-Serum Medium) in a sterile tube.
4. In a separate tube, combine ___µl packaging plasmid premix and ___µl transfer plasmid DNA encoding the gene of interest (GOI). Mix gently by pipetting.
5. Transfer the total volume of packaging premix + transfer plasmid mixture to the tube containing serum-free medium. Mix gently by pipetting.
6. Add ___µl of *Trans*IT-VirusGEN® Reagent. Mix gently by pipetting.
7. Incubate at room temperature for 10-15 minutes to allow transfection complexes to form.

C. Distribute complexes to cells in complete growth medium

1. Add *Trans*IT-VirusGEN® Reagent:DNA complexes to cultured cells (prepared in Step B).
2. Shake cultures on an orbital shaker (e.g. 125 rpm when using a shaker with a 1.9 cm orbital throw) at appropriate temperature and CO_2 levels (e.g. 37°C, 5-8% CO_2, shaking).
3. Incubate transfected cultures for 48 hours prior to lentivirus harvest.

D. Virus Harvest

1. Following the 48-hour incubation, centrifuge cells in a sterile tube at 300 x g for 5 minutes. DO NOT dispose of the supernatant following centrifugation.
2. Transfer the virus containing supernatant into a new sterile tube.
3. Filter through a 0.45 um PVDF filter (e.g. Millipore Steriflip-HV) to remove any cell debris.
4. Immediately flash-freeze aliquots of lentivirus in cryo-tubes and store at –80°C.

Table 4. Volume scaling worksheet for lentivirus generation using *Trans*IT-VirusGEN® Reagent.

Starting conditions per milliliter of complete growth medium (Lentivirus Generation)					
	Per 1 ml		Total culture volume		Reagent quantities
Serum-free Complex Medium	0.1 ml	×	_____ml	=	_____ml
Transfer Plasmid DNA (1 µg/µl stock)	0.5 µl	×	_____ml	=	_____µl
Packaging DNA Premix (1ug/ul stock)	0.5 µl	×	_____ml	=	_____µl
*Trans*IT-VirusGEN® Reagent	3 µl	×	_____ml	=	_____µl

NOTE: *Trans*IT-VirusGEN® Transfection Reagent was optimized using a pre-mix of lentivirus packaging vectors. If using individual packaging plasmids, we recommend a starting ratio of 4 µg *gag-pol* vector, 1 µg *rev* vector and 1 µg VSV-G vector. Premix the packaging plasmids with an equal amount of the transfer vector (e.g. 6 µg) to maintain a 1:1 (wt:wt) ratio of packaging to transfer plasmids.

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