gibco

AAV-MAX Helper-Free AAV Production System

USER GUIDE

for use with:

Viral Production Cells 2.0, Viral Production Medium, AAV-MAX Transfection Kit, Viral-Plex™ Complexation Buffer, AAV-MAX Lysis Buffer

Catalog Numbers A49784, A51218, A4817901, A4817902, A4817903, A50515, A50516, A4983901

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Revision	Date	Description
B.0	28 February 2023	Updated thaw and cryopreservation protocols.
A.0	10 September 2021	New document for AAV-MAX Helper-Free AAV Production System.

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

The Gibco™ AAV-MAX Helper-Free AAV Production System is a high-yield Adeno-Associated Virus (AAV) production system based on a clonal HEK293F-derived cell line adapted to the chemically-defined viral production medium in suspension format. The AAV-MAX Helper-Free AAV Production System includes cells, medium, transfection reagent, transfection booster, production enhancer, and lysis buffer to produce scalable, high-titer adeno-associated viral (AAV) vectors.

Contents and storage

Table 1 AAV-MAX System Components

Contents	Cat. No.	Amount	Storage
Gibco™ Viral Production Cells 2.0[1]	A49784	1 vial (1 × 10^7 cells/mL)	Liquid nitrogen[2]
	A51218	6 × 1 vial	
Gibco™ Viral Production Medium	A4817901	1 L bottle	2°C to 8°C Protect from light
	A4817902	6 × 1 L bottle	
	A4817903	10 L bag	
Gibco™ AAV-MAX Transfection Kit • Gibco™ AAV-MAX Transfection Reagent • Gibco™ AAV-MAX Transfection Booster • Gibco™ AAV-MAX Enhancer	A50515	for 1 L culture	
	A50516	for 10 L culture	
Gibco™ Viral-Plex™ Complexation Buffer	A4983901	100 mL	
Gibco™ AAV-MAX Lysis Buffer	A50520	100 mL	
Gibco™ GlutaMAX™ Supplement	35050061	100 mL	2°C to 8°C

[1] Cells are cryopreserved in 90% Viral Production Medium and 10% DMSO.

[2] Store the frozen cells in liquid nitrogen immediately upon receipt and until ready to use. Do not store the cells at –80°C.

System components

Viral Production Cells 2.0

Gibco™ Viral Production Cells 2.0 (VPCs 2.0) are a clonal derivative of the HEK293F cell line and have been adapted to suspension, high-density culture in Gibco™ Viral Production Medium. These cells can be thawed directly into Gibco™ Viral Production Medium.

Cell line characteristics:

- Transformed via culture with sheared human adenovirus 5 DNA
- Does not contain the SV40 large T antigen
- Cell doubling time of <24 hours
- Achieves maximum cell densities of $>1.2 \times 10^7$ cells/mL in shaker flask cultures
- Maximal AAV production can be achieved between cell passages 3 to 25

Viral Production Medium

Gibco™ Viral Production Medium is a chemically-defined, serum-free, protein-free, animal origin-free medium developed for growth and transfection of VPCs 2.0. Before use, the medium requires supplementation with 4 mM GlutaMAX™ Supplement.

AAV-MAX Transfection Reagent

Gibco™ AAV-MAX Transfection Reagent is a chemically-defined, cationic lipid-based reagent that has been uniquely designed for high efficiency co-transfection of multiple plasmids DNA into high-density VPCs 2.0 with low toxicity.

AAV-MAX Transfection Booster

Gibco™ AAV-MAX Transfection Booster is a chemically-defined reagent that has been uniquely designed to boost efficiency of co-transfection of multiple plasmids DNA into high-density VPCs 2.0. AAV-MAX Transfection Booster works together with AAV-MAX Transfection Reagent to deliver superior transfection efficiency for high-titer AAV vector production.

AAV-MAX Enhancer

Gibco™ AAV-MAX Enhancer is a chemically-defined, serum-free, protein-free formulation that is designed to enhance AAV vector production in VPCs 2.0.

Viral-Plex™ Complexation Buffer

Gibco™ Viral-Plex™ Complexation Buffer is a chemically-defined, serum-free, protein-free, phenol-red free formulation that is designed to facilitate DNA complexation with transfection reagent during DNA transfection.

AAV-MAX Lysis Buffer

Gibco™ AAV-MAX Lysis Buffer is a ready-to-use, chemically-defined, Polysorbate 20-based cell lysis reagent for the extraction of AAV particles from producer HEK293 cells. The buffer is supplied as a 10X solution that can be directly added to HEK293 AAV production cultures to induce cell lysis.

2 Culture Viral Production Cells 2.0

Thaw and establish Viral Production Cells 2.0

Guidelines for handling cells

- **IMPORTANT!** Store the frozen cells in liquid nitrogen immediately upon receipt until ready to use. Do not store the cells at –80°C.
- Avoid subjecting cells to short-term, extreme temperature changes.
- Store cells in liquid nitrogen following receipt on dry ice.
- Allow the cells to remain in liquid nitrogen for 3 to 4 days before thawing.
- For all cell manipulations, mix cells by gentle swirling and avoid vigorous shaking/pipetting.
- For routine cell culture maintenance, subculture cells every 3 to 4 days when the viable cell density reaches 4 to 6 × 10^6 cells/mL. Do not subculture cells that have not reached early log phase growth of ≥4 × 10^6 cells/mL.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
125-mL Erlenmeyer flasks (e.g., Nalgene™ Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile	4115-0125
Orbital shaker: (e.g. MaxQ™ HP Tabletop Orbital Shaker)	SHKE416HP
Temperature and CO_2 controlled incubator (e.g. Large-Capacity Reach-In CO_2 Incubator)	MLS
Reagents and equipment to determine cell viability (e.g., hemocytometer with trypan blue or cell counter)	MLS

Thaw Viral Production Cells 2.0

1. Add 30 mL of pre-warmed Viral Production Medium supplemented with 4 mM GlutaMAX™ Supplement to a 125-mL Erlenmeyer shaker flask.

   Note: GlutaMAX™ Supplement, Cat. No. 35050061 is formulated at 200 mM.

2. Remove a vial of Viral Production Cells 2.0 from liquid nitrogen and swirl gently in a 37°C water bath for 1 to 2 minutes to thaw the cells rapidly until only a small amount of ice remains.

   Note: Do not submerge the vial in the water.

3. Just before the cells are completely thawed, decontaminate the vial with 70% ethanol before opening it in a laminar flow hood.

4. Gently invert the cell vial to mix the contents. Uncap the cell vial and transfer 50 µL of cells into 450 µL of Ca^{2+}/Mg^{2+} free PBS for viability and viable cell density determination by trypan blue dye exclusion assay.

   Note: Trypan blue may interact with components in cell culture media leading to aggregation that can be misinterpreted as dead cells using typical cell counting instruments and algorithms. Dilution with PBS is not required during routine cell culture maintenance.

5. Using a 1-, 2-, or 5-mL pipette, gently transfer the remaining cell volume drop wise to the shake flask containing the pre-warmed culture medium prepared in step 1.

6. Incubate the cells in a 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker platform. The use of non-humidified incubators is not recommended due to the significant loss of volume in the culture flasks by evaporation.

   Note: Set the shake speed to 125 ±5 rpm for shakers with a 19-mm shaking diameter, 120 ±5 rpm for shakers with a 25-mm shaking diameter and 95 ±5 rpm for shakers with a 50-mm shaking diameter.

7. Three to four days post-thaw, determine the viable cell density and percent viability. Cell viability should be ≥90% with a viable cell density >1 × 10^6 viable cells/mL.

   Note: If the viability is <90% on days 3 to 4 post-thaw, cells may be cultured for up to an additional 3 days in order to reach the desired viability. Cells should not be subcultured until viable cell density reaches >1 × 10^6 viable cells/mL.

8. For subsequent routine cell culture maintenance, subculture cells every 3 to 4 days when the viable cell density reaches 4 to 6 × 10^6 viable cells/mL according to **Table 4**.

   Note: Do not subculture cells before reaching early log phase growth of ≤4 × 10^6 cells/mL. Similarly, do not let cells overgrow above ≥6 × 10^6 cells/mL. Modify the initial seeding density to attain target cell density of 4–6 × 10^6 viable cells/mL at the time of subculturing.

Subculture Viral Production Cells 2.0

VPCs 2.0 are capable of achieving high cell densities; therefore, it is important that cells attain a minimum density of 4 to 6 × 10^6 viable cells/mL at the time of subculturing.

1. At the time of subculture, calculate viable cell density.

   Note: If using a Vi-CELL™ cell counting instrument, for the recommended settings for this cell line see Table 2 and Table 3.

2. Use the viable cell density to calculate the volume of cell suspension required to seed a new shaker flask according to the recommended seeding densities in Table 4 and the recommended culture volumes in Table 5.

Table 2 Recommended Vi-CELL™ XR cell counting settings

Parameter	Value	Parameter	Value
Minimum diameter	10	Cell brightness	85
Maximum diameter	30	Cell sharpness	100
Number of images	50	Viable cell spot brightness	65
Aspirate cycles	3	Viable cell spot area	5
Trypan blue mixing cycles	3	Minimum circularity	0
Decluster degree	Medium	—	—

Table 3 Recommended Vi-CELL™ BLU cell counting settings

Parameter	Value	Parameter	Value
Minimum diameter	10	Cell sharpness	7.0
Maximum diameter	30	Viable cell spot brightness	40
Number of images	100	Viable cell spot area	5
Aspirate cycles	6	Maximum cirularity	0.10
Trypan blue mixing cycles	6	—	—
Decluster degree	High	—	—

Table 4 Recommended seeding densities for routine cell culture maintenance

Sub-culture timing	Recommended seeding density
For cells ready 3 days post-subculture	0.6 × 10^6 viable cells/mL
For cells ready 4 days post-subculture	0.3 × 10^6 viable cells/mL

Note: Modify the initial seeding density to attain the target cell density of 4 to 6 × 10^6 viable cells/mL at the time of subculturing.

Table 5 Recommended volumes for routine cell culture maintenance

Flask size	Culture volume[1]	Shake speed
125 mL	30 mL	125±5 rpm (19-mm orbital diameter) 120±5 rpm (25-mm orbital diameter) 95±5 rpm (50-mm orbital diameter)
250 mL	60 mL	
500 mL	120 mL	
1 L	240 mL	
2 L	480 mL	
2.8 L	700–1000 mL	90±5 rpm (19-mm orbital diameter) 85±5 rpm (25-mm orbital diameter) 80±5 rpm (50-mm orbital diameter)

[1] If using volumes outside of the recommended range, it is critical to ensure that all cell growth (i.e., doubling times), health (i.e., cell diameter, viability), and production levels remain consistent with control conditions. Cell performance is decreased if cell health is compromised.

3. Transfer the appropriate number of cells to fresh, pre-warmed Viral Production Medium supplemented with 4 mM GlutaMAX™ Supplement in a shaker flask.

4. Incubate the flasks in a 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker platform until the cultures reach a density of 4 to 6 × 10^6 viable cells/mL.

Note: Cells that are subcultured at densities outside of this early log-phase growth window may show longer doubling times and lower titers over time. Modify the initial seeding density to attain the target cell density of 4 to 6 × 10^6 viable cells/mL at the time of subculturing.

5. Repeat step 1 to step 3 to maintain or expand cells for transfection.

Cryopreserve Viral Production Cells 2.0

Viral Production Cells 2.0 can be frozen directly in Viral Production Medium with 4 mM GlutaMAX™ Supplement with 7.5% DMSO. Alternatively, conditioned cryopreservation medium consisting of 42.5% fresh Viral Production Medium, 50.0%, conditioned medium and 7.5% DMSO can be used.

Note: It is critical that chemical compatibility be maintained throughout the freezing process to eliminate the potential for plastics leachables/extractables to negatively impact cell health. For all steps where DMSO is present (with exception only to the pipet tips used for aliquoting the final cell solution into cryo-vials), glass serological pipettes are suggested to be used, as polystyrene is generally not compatible with concentrated DMSO. Similarly, DMSO containing freeze medium must be prepared in polypropylene or other known DMSO compatible bottles and the final cell suspension in freeze medium must be prepared in polypropylene or other known DMSO compatible bottles prior to aliquoting into polypropylene freeze tubes. Additionally, all pipets and bottles may be rinsed with

sterile water, PBS or culture medium before use as desired. Refer to **https://tools.thermofisher.com/content/sfs/brochures/D20480.pdf** for guidance on chemical compatibility.

1. Determine the density and volume of cells required for banking cells at a final density of 1×10^7 viable cells/mL in 1.1 mL total volume. Expand the cells, maintaining a viable cell density of $4–6 \times 10^6$ viable cells/mL, until the desired volume of cells to be banked is ready. Do not use shake flasks larger than 2 L to culture the cells, as these flasks differ in shape and require altered shaking speeds and be sure to adhere to the shaking speed, orbital diameter and maximum flask volume recommendations.

2. While expanding the cells for banking, prepare an additional flask which will be used to generate conditioned medium. This flask should be prepared and expanded in the same manner as the flasks used for cell banking.

   Note: Based on the total volume of the bank, prepare at least ½ volume conditioned medium (i.e., If total volume for cell banking is 100 mL, at least 50 mL of conditioned medium will be required).

3. Prepare labels and label the appropriate number of vials. If vials are labeled on a day other than that of the harvest, store vials in a biosafety cabinet.

   Note: To reduce the risk of damaging the cells during freezing procedures, cells pellets will be resuspended in cold, 100% conditioned medium followed by 1:1 addition of cold freeze medium with 15% DMSO to reach a final concentration of 7.5% DMSO, 50% conditioned medium, and 42.5% fresh medium. It is critical to resuspend the cell pellets in cold medium free of DMSO.

   Note: If use of 100% fresh culture medium for banking cells is desired, cells pellets will be resuspended in cold, 100% fresh medium followed by a 1:1 addition of cold, 15% DMSO in fresh cell culture medium to reach a final concentration of 7.5% DMSO and 92.5% fresh medium.

Prepare conditioned medium

All conditioned medium is to be pre-chilled before use in cell banking.

1. Remove conditioned medium flask from incubator and transfer the entire volume of cell suspension into a sterile polypropylene centrifuge tube or bottle.

2. Centrifuge the cells at 300 × *g* for 10 minutes at 2–8°C.

3. Carefully decant the supernatant into a sterile polypropylene bottle without disturbing the cell pellet; the decanted supernatant will be used as the conditioned medium.

4. Store the conditioned medium in a 2–8°C refrigerator or on ice for a minimum of 2 hours.

5. Discard the cell pellets.

Prepare freeze medium (2X)

1. In a sterile polypropylene bottle, prepare the required amount of fresh culture medium supplemented with 15% DMSO. This represents a 2X freeze medium. It is recommended to use glass serological pipettes for transferring the concentrated DMSO to the culture medium. Keep 2X DMSO freeze medium cold at 2–8°C or on ice until use.

2. Remove calculated volume of cells from incubation and transfer into sterile polypropylene centrifuge bottle/tube. Centrifuge the cells at 200 × *g* for 10 minutes at 2–8°C. Carefully decant the supernatant without disturbing the cell pellets. After removing the supernatant, gently flick the bottom of the centrifuge tube to loosen the cell pellet.

3. Resuspend the cell pellet by gently pipetting with ~10% volume of conditioned medium using a wide bore pipet (i.e., If total bank volume is 200 mL, use 20 mL to resuspend cell pellet).

4. Add additional conditioned medium to the centrifuge bottle to obtain a 2X cell stock. For example, if banking at 1×10^7 cells/mL prepare a 2X cell stock at 2×10^7 cells/mL. Gently swirl the bottle to ensure a homogenous mixture and keep cold on ice or cold blocks.

   Note: It is critical that the next steps are performed as quickly as possible to limit the DMSO exposure time.

5. Using a glass serological pipette, add the calculated volume of cold, 2X freeze medium to the bottle containing 2X cell stock in conditioned medium.

6. Gently swirl the bottle to ensure a homogenous mixture and keep cold on ice or cold blocks.

   At this point the volume should be equal to the total bank volume at a final concentration of 7.5% DMSO, 50% conditioned medium, and 42.5% fresh medium with a cell density of 1×10^7 cells/mL.

7. Immediately dispense a 1.1 mL aliquot of the final cell suspension from step 6 into labeled cryo-vials using a repeater pipette or sterile serological pipettes.

8. Gently swirl the cell suspension to mix before each refill of the multi-dispenser pipette or serological pipette. Make sure to keep the cell suspension cold during the entire aliquoting process.

   Note: The DMSO in the 2X freeze medium is harmful to the cells so it is important to limit the amount of DMSO exposure prior to freezing. We recommend keeping the DMSO exposure time ≤ 60 minutes as possible and keeping all reagents cold during this time.

9. Transfer the cryo-vials to isopropanol containing cryo-freezing chambers and store at -80°C for 24–48 hours. Do not store cells at -80°C for more than 48 hours. After 24–48 hours transfer cells to final storage in vapor phase liquid nitrogen.

   This freezing regimen approximates a 1°C per minute cooling in the isopropanol containers to -80°C followed by a 2°C per minute cooling in vapor phase liquid nitrogen to final storage temperature. These cooling conditions may be utilized as a basis for controlled rate freezing protocol design.

3 Transfect Viral Production Cells 2.0

Transfection guidelines

- Allow freshly thawed cells to recover in culture for three or more passages post-thaw and before transfection.
- During all cell manipulations, mix the cells by gentle swirling; avoid vigorous mixing/pipetting. Cell health is critical to maximal performance.
- Gently invert the Transfection Reagent 4 to 5 times before use to ensure thorough mixing.
- Complexation of plasmid DNA and Transfection Reagent takes place at room temperature.
- See Table 7 for transfection at various scales.

Equipment guidelines

- For optimal performance, it is important to follow the recommended shaking diameter, shaking speed, flask size/type, and volume of culture to be transfected provided in this protocol.
- Humidified incubators (≥80% relative humidity) are recommended to reduce evaporation during AAV production runs. When using multi-well plates, use high-humidity settings if available, as evaporation can introduce variation and impact results.
- Ensure equipment is calibrated for temperature. In some instances, the total heat from the incubator and the shaker can cause cell culture temperatures to exceed the recommended ranges and lead to decreased cell growth, clumping or cell death. In such instances, reduce the temperature setting of the incubator to compensate for heat generated by the shaker.
- Ensure that equipment is calibrated for CO_2. Levels of CO_2 should not exceed 8%.

Required materials

- Viral Production Cells 2.0 cultured in Viral Production Medium supplemented with 4 mM GlutaMAX™ Supplement
- User-specific plasmids: Transfer plasmid (containing the gene of interest), Rep/Cap plasmid, Helper plasmid.
- AAV-MAX Transfection Kit
- Viral-Plex™ Complexation Buffer
- Viral Production Medium supplemented with 4 mM GlutaMAX™ Supplement, pre-warmed to 37°C
- Disposable, sterile Erlenmeyer flasks
- Orbital shaker in a 37°C incubator with ≥80% relative humidity and 8% CO_2
- Reagents and equipment to determine viable cell density and percent viability

Optimized transfection conditions

Table 6 Reagents required for AAV production

Condition	Amount
Viral-Plex™ Complexation Buffer	100 µL per mL of culture volume to transfect
Plasmid DNA[1]	1.5 µg per mL total plasmid DNA of culture volume to transfect (i.e. Transfer plasmid + Rep/Cap plasmid + Helper plasmid)
AAV-MAX Transfection Reagent	6 µL per mL of culture volume to transfect
AAV-MAX Transfection Booster	3 µL per mL of culture volume to transfect
AAV-MAX Enhancer	10 µL per mL of culture volume to transfect
AAV-MAX Lysis Buffer	100 µL per mL of culture volume to transfect
Viral Production Cells 2.0	3×10^6 cells/mL density at transfection

[1] Optimizing the molar ratio of plasmids based on plasmid size is highly recommended. An example calculation for appropriate amounts of the 3 plasmids based on plasmid size is depicted in Table 16.

Table 7 Recommended reagent volumes for transfection at various scales

	Vessel type				
	125 mL flask	**250 mL flask**	**1 L flask**	**2 L flask**	**2.8 L flask**
Number of cells required	90×10^6	180×10^6	720×10^6	$1{,}440 \times 10^6$	$3{,}000 \times 10^6$
Culture volume to transfect	30 mL	60 mL	240 mL	480 mL	1,000 mL
Shake speed [1]	125 ± 5 rpm (19 mm orbital diameter) 120 ± 5 rpm (25 mm orbital diameter) 95 ± 5 rpm (55 mm orbital diameter)				95 ± 5 rpm 90 ± 5 rpm 55 ± 5 rpm
Amount of plasmid DNA	1.5 µg total plasmid DNA per mL of culture to transfect				
Volume of plasmid DNA [2]	45 µL	90 µL	360 µL	720 µL	1.5 mL
Viral-Plex™ Complexation Buffer to dilute plasmid DNA [3]	3 mL	6 mL	24 mL	48 mL	100 mL
AAV-MAX Transfection Reagent	180 µL	360 µL	1.44 mL	2.88 mL	6 mL
AAV-MAX Transfection Booster	90 µL	180 µL	720 µL	1.44 mL	3 mL
AAV-MAX Enhancer	300 µL	600 µL	2.4 mL	4.8 mL	10 mL
Final culture volume	~34 mL	~68 mL	~272 mL	~544 mL	~1,120 mL

[1] Recommended shake speed ranges: optimal shake speed should be determined empirically based on the specific laboratory equipment used.
[2] Assuming a plasmid DNA concentration of 1 mg/mL and a final concentration of 1.5 µg/mL of plasmid DNA.
[3] Volume used to dilute plasmid DNA.

Transfection procedure

For volumes for transfection at various scales, see Table 7.

Subculture and expand cells until cells reach a density of approximately 4 to 6 × 10^6 cells/mL.

Day 0: Prepare and transfect cells

1. On the day of transfection (Day 0), determine viable cell density and percent viability. Cells should have reached a density of approximately 4.5 to 6.0 × 10^6 viable cells/mL. Viability should be ≥95% to proceed with transfection.

2. Dilute the cells from step 1 to a final density of 3 × 10^6 viable cells/mL with fresh Viral Production Medium supplemented with 4 mM GlutaMAX™ Supplement.

3. Immediately add AAV-MAX Enhancer (i.e. add 300 µL to transfect 30 mL of cells). Swirl the flask(s) gently to mix the cells. Incubate the cells in a 37°C incubator with a humidified atmosphere of 8% CO_2 on an orbital shaker until the DNA/transfection complexation process is completed (step 5 to step 7). For suggested shake speeds, see Table 7.

4. Prepare transfer plasmid DNA, Rep/Cap plasmid DNA and helper plasmid DNA.
   a. Use 1.5 µg /mL of total plasmid DNA to the culture volume to be transfected (See Table 7 for the recommended volumes at various scales).

   Note: Plasmid DNA molar ratio optimization based on plasmid size is highly recommended based on the desired serotype and transfer plasmid information. See Appendix A, “Plasmid ratio optimization” for example of ratio optimization of 3 plasmids DNA.

5. Prepare transfection complexes as described (See Table 7 for the recommended volumes at various scales).
   a. Gently invert the AAV-MAX Transfection Reagent bottle 4 to 5 times to mix.

   b. Dilute total plasmid DNA from step 4 (e.g., 45 µg total DNA to transfect a 30 mL culture) with Viral-Plex™ Complexation Buffer to a final volume of 10% of the culture volume to be transfected (e.g., 3 mL Viral-Plex™ Complexation Buffer to transfect a 30 mL culture).

   c. Mix by swirling gently.

   d. To a new tube, add neat AAV-MAX Transfection Booster at 3 µL per mL of culture to be transfected (e.g., 90 µL AAV-MAX Transfection Booster to transfect a 30 mL culture) followed by neat AAV-MAX Transfection Reagent at 6 µL per mL of culture to be transfected (e.g., 180 µL AAV-MAX Transfection Reagent to transfect a 30 mL culture).

   e. Mix by gentle pipetting (do not vortex).

   Note: The AAV-MAX Transfection Booster/AAV-MAX Transfection Reagent complex is stable for up to 1 hour at room temperature or 2 days at 2–8°C.

f. Add the pre-mixed AAV-MAX Transfection Booster and AAV-MAX Transfection Reagent (e.g., 90 µL AAV-MAX Transfection Booster + 180 µL AAV-MAX Transfection Reagent) from substep 5e to the diluted plasmid DNA from substep 5c. Mix by swirling, inversion or gentle pipetting 2 to 3 times (do not vortex).

6. Incubate plasmid DNA/AAV-MAX Transfection Booster/ AAV-MAX Transfection Reagent complexes from substep 5f at room temperature for 20 to 30 minutes, then gently transfer the solution to the shaker flask(s) prepared in step 3.

   Optimization of the incubation time of the plasmid DNA/AAV-MAX Transfection Booster/AAV-MAX Transfection Reagent complexes can be performed to increase titers. This is particularly recommended for small or large plasmid sizes.

7. Incubate the cells in a 37°C incubator with a humidified atmosphere of 8% CO_2 on an orbital shaker for approximately 72 hours. For suggested shake speeds, see Table 5.

Harvest AAV particles

Handling of AAV particles must be performed as per institutional guidelines. All materials that come into contact with AAV solution should be appropriately disinfected prior to disposal.

The AAV-MAX Lysis Buffer is a 10X formulation that can be added directly to the cell culture to induce lysis.

Harvest AAV particles 70 to 72 hours post-transfection.

1. Add AAV-MAX Lysis Buffer directly to the culture flask at a 1:10 dilution (e.g., 3.3 mL of AAV-MAX Lysis Buffer to a 30 mL culture volume) and swirl the flask to evenly distribute the lysis buffer.

   Note: If performing qPCR measurement of titers, remove 5 mL from the culture flask after performing step 1 and perform lysis by incubating at 37°C for 1 hour at 250 rpm on an orbital shaker. Proceed to step 3.

   Note: If proceeding to a downstream workflow, add $MgCl_2$ (final concentration: 2 mM) and Benzonase (final concentration: 90 U/mL) to the remaining culture in the flask from step 1, then proceed to step 2.

2. Incubate the flask at 37°C for at least 2 hours on an orbital shaker (for suggested shake speeds, see Table 5).

   Note: Once cells are lysed, the culture will appear to contain visible cell debris.

   Note: Lysis incubation times can vary based on AAV serotype and production scale. Therefore, it is recommended to optimize lysis conditions for your experiments prior to moving to large scale.

3. Transfer the cell lysate to an appropriate flask or tube, then centrifuge at 4°C at 13,000 × *g* for 10 min for smaller volumes (microfuge scale) or at 4,500 × *g* for 30 minutes for larger scale of production.

4. Transfer the supernatant containing crude AAV particles to an appropriate storage container.

5. For qPCR titer measurement, transfer 50 to 100 µL of lysate to a 96-well plate.

   See Chapter 4, “AAV titer measurement by qPCR”.

Note: It is recommend that multiple replicates for each sample are prepared to account for qPCR assay variation.

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6. Harvested crude AAV particles can be stored at -80°C for long term storage.

Note: To avoid repeated freeze/thaw cycles, virus aliquoting is highly recommended. To thaw AAV samples, bring the tube to room temperature and mix the AAV sample by gentle pipetting or inverting. Do not vortex or avoid mixing vigorously.

Note: Crude AAV vectors can temporarily be stored at 4°C for short duration (i.e. overnight). If samples are stored for extended periods of time at 4°C, precipitation can occur. This is dependent on AAV serotype and/or production scale. If precipitation is observed, reclarify the samples prior to proceeding to the next step.

Note: To disinfect AAV properly, prepare a 10% bleach solution, then disinfect used pipette tips, serological pipettes and culture flasks before disposal. To discard remaining AAV samples, add the 10% bleach solution directly to the AAV solution, then incubate for a minimum of 30 minutes before disposal.

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4 AAV titer measurement by qPCR

The AAV titration protocol can be used to determine the number of genome-containing particles in AAV preparations. The qPCR method for AAV titer measurement requires design of primers and probe based on the Gene of Interest (GOI). For this protocol, crude AAV lysate with GFP as the GOI is described for general guidelines. Note that the qPCR set up will need to be designed and optimized based on user's GOI.

Required materials

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Item	Source
Linearized Transfer plasmid for qPCR standard curve (1.0×10^{10} copies/µL)	—
DNase I	18047019
Exonuclease I	EN0582
Recombinant Proteinase K Solution	AM2548
DNase I Buffer (10X)	AM8170G
Platinum™ Quantitative PCR SuperMix-UDG w/ROX™	11743500
GFP specific primers (Forward/Reverse) mix (10 µM)	—
GFP specific TaqMan™ probe (FAM/TAMRA 3 µM)	—
UltraPure™ DNase/RNase-Free Distilled Water	10977015
Adhesive PCR Plate Seals	AB0558
MicroAmp™ Optical Adhesive Film	4311971
PBS	14190144
TE, pH 8.0, RNase-free	AM9849
Pluronic™ F-68 Non-ionic Surfactant (100X)	24040032
2X Proteinase K Buffer	See Table 8

Table 8 2X Proteinase K Buffer

Reagent	Final concentration	Stock concentration	Volume
$MgCl_2$	1 mM	1 M	0.1 mL
$CaCl_2$	1 mM	1 M	0.1 mL
Tris_HCl pH 8	20 mM	1 M	2 mL
Na Deoxycholate	0.02%	1%	2 mL
SDS	0.01%	1%	1 mL
DNase/RNase Free Water	—	1%	94.8 mL
Total volume	—	—	**100 mL**

Procedural guidelines

- For best results, perform DNase I/ Exonuclease I, Proteinase K and qPCR steps on the same day. If you need to stop, complete DNase I/ Exonuclease I and Proteinase K step and store at 4°C overnight then continue next day.
- Prior to the start of the titering QC process, thaw all the reagents on ice.
- Use aerosol filter pipette tips, a designated clean work area, DNase/RNase free water.

qPCR AAV titer measurement

Prepare DNase I/Exonuclease I digestion

1. For qPCR procedure sample preparation, aliquot the harvested crude AAV lysates into 96-well PCR plate in triplicates per sample.

2. Dilute crude AAV lysates by 1:10 with PBS or 0.01% Pluronic™ F-68 solution in PBS. Mix samples by pipetting at least 10 times prior to setting up DNase I/Exonuclease I digestion (i.e. 10 µL sample with 90 µL PBS/0.01% Pluronic™ F-68 solution).

   Dilution of samples for DNaseI/Exonuclease I digestion is recommended to ensure complete unpackaged DNA digestion and accurate titer measurement.

3. Prepare master mix for DNase I/Exonuclease I digestion set up.

Table 9 Reaction components per well (96-well)

Component	Reaction per well
Crude AAV lysate	5 µL
DNase I Buffer (10X)	5 µL
DNase I (100 U/ reaction)[1]	0.5 µL
Exonuclease I (1 U/ reaction)[2]	0.05 µL
DNase/RNase free water	39.45 µL
Total volume	**50 µL**

[1] DNase I: 212.9 U/µL
[2] Exonuclease I: 20 U/µL

4. Transfer the prepared master mix into a reservoir and add 45 µL per well into a 96-well PCR plate. Then add 5 µL of crude AAV lysates that has prepared in step 1.

5. Mix samples by pipetting at least 10 times or tightly seal the plate and vortex briefly.

6. Seal the plate with the adhesive film and centrifuge down the plate at 2400 × *g* for 1 min.

7. Run the DNase I/Exonuclease I digestion process on a thermal cycler.

Table 10 Thermal cycler set up

Stage	Temperature	Time
Stage 1	37°C	60 min
Stage 2	85°C	20 min
Stage 3	4°C	4°C hold

Prepare Proteinase K digestion

1. Prepare a master mix of Proteinase K digestion set up:

Component	Reaction per well
2X Proteinase K buffer	49 µL
Proteinase K (20 mg/mL)	1 µL
Total volume	**50 µL**

2. Take out the DNase I/Exonuclease I digestion plate from the thermal cycler. Briefly spin the plate in a benchtop centrifuge at 2400 × g for 1 min and then carefully remove the adhesive film. To avoid cross contamination, hold the plate firmly while removing the seal.

3. Add 50 µL of Proteinase K master mix on top of the DNase I/Exonuclease I reaction plate. Change tips for each pipetting step to avoid cross contamination.

4. Seal the plate with new adhesive film completely, vortex briefly to mix, and spin down at 2400 × g for 1 min.

5. Run the Proteinase K reaction in a thermal cycler following the settings:

Stage	Temperature	Time
Stage 1	60°C	60 min
Stage 2	95°C	10 min
Stage 3	4°C	Hold

Prepare qPCR samples

1. To prepare a sample dilution plate, add 196 µL of DNase/RNase free water or 1X TE buffer into each well of a new 96-well plate.

   Prepare additional wells for Negative control (no template control) samples for qPCR set up.

2. Remove the Proteinase K plate from the thermal cycler and briefly spin the plate in a benchtop centrifuge at 2400 × *g* for 1 min and then carefully remove the adhesive film. To avoid cross contamination, hold the plate firmly while removing the seal.

3. Add 4 µL of Proteinase K digested sample from step 1.

4. Mix samples by pipetting at least 10 times or seal the plate very tight and vortex briefly, then centrifuge the plate at 2400 × g for 1 min. Samples are ready to use for qPCR, place on the ice until next steps are ready.

Prepare standard curve

1. Prepare 8 of Eppendorf tubes and label on them prior to dilute linearized plasmid as indicated in Table 11.

Table 11 Standard curve dilution series

Tube	Standard	H_2O or 1X TE	Final concentration (DNA copies/µL)
Stock	—	—	1×10^{10}
STD 8	2 µL standard stock	198 µL	1×10^{8}
STD 7	20 µL STD 8	180 µL	1×10^{7}
STD 6	20 µL STD 7	180 µL	1×10^{6}
STD 5	20 µL STD 6	180 µL	1×10^{5}
STD 4	20 µL STD 5	180 µL	1×10^{4}
STD 3	20 µL STD 4	180 µL	1×10^{3}
STD 2	20 µL STD 3	180 µL	1×10^{2}
STD 1	20 µL STD 2	180 µL	1×10^{1}

2. Add 198 µL of DNase/RNase free water or 1X TE buffer to the tube STD-8 and 180 µL to the tubes from STD-7 to STD-1.

3. Add 2 µL of prepared linearized DNA (1.0×10^{10} copies/µL) to make 1.0×10^{8} copies/µL of linearized transfer plasmid to #8 tube (STD-8), then vortex to mix and spin down.

4. Repeat the dilution process to complete the 10-fold serial dilution.

5. Aliquot 25 µL (N=3) of each prepared Standard sample on to a new 96-well plate as in Table 12.

Table 12 qPCR Standard sample on 96-well plate (N=3)

1	2	3	4	5	6	7	8	9	10	11	12
STD 8	STD 8	STD 8	STD 7	STD 7	STD 7	STD 6	STD 6	STD 6	STD 5	STD 5	STD 5
STD 4	STD 4	STD 4	STD 3	STD 3	STD 3	STD 2	STD 2	STD 2	STD 1	STD 1	STD 1

Prepare qPCR reaction (384-well plate)

1. Prepare master mix for qPCR reaction set up

Table 13 Reaction components per well (384-well)

Component	Reaction per well
Sample	3 µL
Platinum™ Quantitative PCR SuperMix-UDG w/ROX™	7.5 µL
GOI specific probe	0.11 µL
GOI specific primer F/R	1.13 µL
DNase/RNase free water	3.26 µL
Total volume	**15 µL**

2. Transfer the prepared master mix into a reservoir and add 12 µL per well into a 384-well qPCR plate.

3. Add 3 µL of qPCR standard sample that was prepared on Table 11. Each time add the sample into every other well to make duplicate per well.

   For example, qPCR Standard Sample A1 (96-well plate) will be loaded into A1 and A2 of 384-well plate. Rows A and B of the 384-well qPCR plate will be Standard samples as described in Table 14. With triplicates of enzyme digestions and duplicates of qPCR reaction per sample, total 6 replicates will be ready per sample.

Table 14 qPCR Standard sample on 384-well plate (N=2 for qPCR, total N=6 per sample)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	STD-8						STD-7						STD-6						STD-5					
B	STD-4						STD-3						STD-2						STD-1					
C	AAV samples						—						—						—					

4. In the same way, add 3 µL of diluted AAV samples from “Sample Dilution Plate” to 384-well qPCR plate.

5. Seal the plate tight with optical adhesive film and vortex for 30 sec to mix. Spin down the plate at 2400 × *g* for 5 min.

6. Set up qPCR following program in Table 15. For TaqMan™ probe, choose **FAM** for Reporter and **TAMRA** for Quencher. Define the sample position based on the prepared 384-well qPCR plate.

Note: If you are using QuantStudio™ 6 or 7, select **None** for Passive Reference.

Table 15 qPCR cycling set up

Stage	Temperature	Time
Stage 1	50°C	2 min
Stage 2	95°C	2 min
Stage 3 (40 cycles)	95°C	15 sec
	60°C	1 min

7. Load the plate into the qPCR machine and run.

Data analysis

1. To calculate the titer based on the Ct values of the standard, calculate the following values:

- Standard curve slope
- Standard curve intercept
- E_{AMP} (optimum qPCR amplification efficiency is 95–105%: E_{AMP}= 1.95–2.05)

2. Vg for 3 µL of qPCR sample= E_{AMP} ^(STD curve intercept- Ct of sample)

3. vg/mL = (3 µL of vg/3*50)*10*2*(1000)/0.9*10

- (Vg for 3 µL /3) = vg/1 µL of diluted sample
- *50 for pre-qPCR dilution step
- *10 for DNase/Exonuclease reaction step
- *2 for Proteinase K reaction step
- *1000 to adjust to per mL
- /0.9 the 1 mL of cell lysate contains 0.9 mL of crude AAV
- *10 for pre-DNase dilution step

4. Calculate the final vg/mL by calculating the average of replicates of one qPCR sample.

A Plasmid ratio optimization

To achieve the highest transfection efficiency for AAV production, molar ratio optimization of the three plasmids (Transfer plasmid, Rep/Cap plasmid and Helper plasmid) based on plasmid size is highly recommended. For AAV production using the AAV-MAX system, 1.5 mg of total DNA is required for 1 L of AAV production. Based on the total number of base pairs (bp) of all 3 plasmids, you can determine total µg/bp that is needed to use for each plasmid.

Table 16 Example calculation of plasmid DNA amounts based on plasmid size

Plasmid	Plasmid size	Molar ratio	Plasmid size considering molar ratio
Transfer Plasmid	5,400 bp	1	5,400 bp
Rep/Cap Plasmid	7,330 bp	2	14,660 bp
Helper Plasmid	11,635 bp	0.5	5,818 bp
Total bp	**24,365 bp**	—	**25,878 bp**

1500 µg/25878 bp = 0.06 µg/bp. The following table summarizes the amount needed for each plasmid:

Plasmid	Total DNA required
Transfer Plasmid	0.06 µg/bp × 5,400 bp = ~325 µg
Rep/Cap Plasmid	0.06 µg/bp × 14,660 bp = ~880 µg
pHelper	0.06 µg/bp × 5,818 bp = ~350 µg

B

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

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Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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