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AAV Production in HEK293T Cells

Addgene the nonprofit plasmid repository[1]

[1]Addgene

1 Works for me dx.doi.org/10.17504/protocols.io.47igzke

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ABSTRACT

This protocol is used for AAV production in HEK293T cells. To see the full abstract and additional resources, visit the Addgene protocol page.

Sample Data

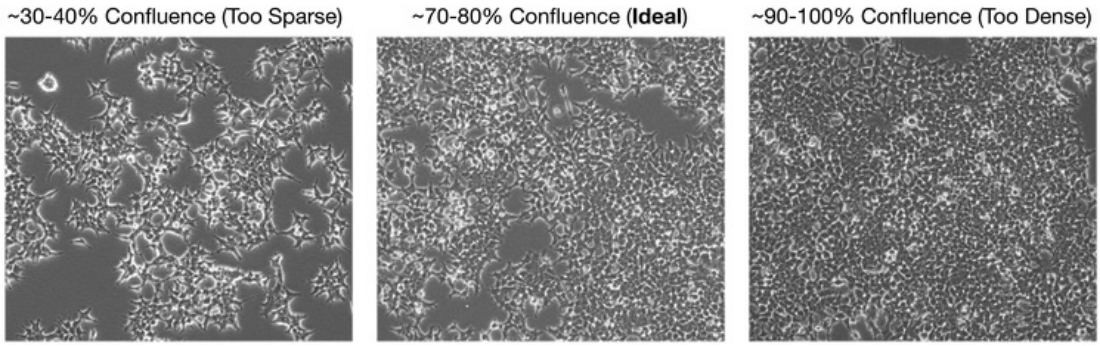

Figure 1: HEK293T cells at various confluencies. This AAV production protocol should be started with cells that are ~80% confluent (center panel). These cells were imaged with a 10X objective and were split 2 days before these images were taken.

EXTERNAL LINK

https://www.addgene.org/protocols/aav-production-hek293-cells/

GUIDELINES

Workflow Timeline

Day 0: Seed cells in CF2

Day 2: Seed cells in CF5

Day 3 (am): Transfect cells

Day 7 (am): Harvest cells

MATERIALS TEXT

Equipment

- Biosafety cabinet

- Pipetman
- Pipettors
- Pipettes
- Incubator
- Sterile stir bars

Reagents

- Adherent HEK293T cells (ideally AAV-293T clones)

Pro-Tip
While adherent, these cells are very loosely attached to the dish surface and should be handled carefully. Avoid touching the cells when replacing media.

- T-150 flask (Corning, 150 cm^2)
- Five chamber cell stack (CF5, Corning, 3180 cm^2)
- Heat-inactivated FBS (HI-FBS)

Pro-Tip
Different brands and lots of FBS can promote or inhibit transfection. Test a variety of brands and lots of FBS to find one suitable for your protocols. FBS can be purchased already heat inactivated or it can be inactivated in the lab by heating to **56 °C** °C for **00:30:00** .

- 0.45 µm polyethersulfone (PES) filter system

Pro-Tip
Do not use filters made of materials other than PES. AAV particles stick to many other surfaces, but do not stick to PES. Using a PES filter will maximize titer.

- DMEM high glucose with L-glutamine and 1 mM pyruvate
- Opti-MEM
- 0.05% Trypsin/EDTA
- 1X PBS pH 7.4
- 1 mg/mL Polyethyenimine (PEI) 25 KDa MW

Pro-Tip
Other transfection reagents may be used in this protocol, but their conditions must be optimized.

- Plasmids for transfection : pHelper ; pRC (Rep-Cap), plasmid expressing your gene of interest
- Triton X-100
- Benzonase / DNAse I
- 40% Polyethylene Glycol 8000 (PEG)
- RNase-free DNAse

Reagent Preparation

DMEM Complete: 10% v/v FBS and 4 mM L-alanyl-L-glutamine. To a **500 ml** bottle of DMEM high glucose, add 55 mL of heat

inactivated FBS and 11 ml of 200 mM L-alanyl-L-glutamine. Store at 4 °C .

1 mg/mL polyethylenimine (PEI) solution:

- Dissolve 100 mg of PEI powder into 100 ml of deionized water.
- While stirring, slowly add hydrochloric acid until the solution clears.
- Check the pH of the solution and use hydrochloric acid or sodium hydroxide to adjust the pH to 7.0.

Pro-Tip
The pH of this solution will drift pretty rapidly upon addition of acid or base. Add only a few drops at a time. Allow them to mix and recheck the pH to prevent over or undershooting the desired pH.

- Allow the solution to mix for 00:10:00 and then recheck the pH to ensure that it has not drifted.
- Filter the solution through a 0.22 µm membrane.
- Aliquot 500-1000 µl into sterile tubes.
- Store the tubes at -80 °C .
- After thawing, the solution can be stored at 4 °C for up to 2 months. After 2 months, discard the tube and thaw a new working stock.

40% POLYETHYLENE GLYCOL (PEG) 8000 solution:

- Dissolve 400 g of PEG 8000 and 24 g of NaCl into ddWater and adjust to a final volume of 1,000 mL.
- Stir at room temperature until fully dissolved.

Pro-Tip
This step is challenging due to the high viscosity of PEG. Stirring under medium heat will promote faster dissolution.

- Adjust the pH to ~7.4.
- Autoclave or sterile filter.

Pro-Tip
Stirring during the cooling period is recommended or the solution may separate into phases.

- Aliquot and store at 4 °C .

SAFETY WARNINGS

See SDS (Safety Data Sheet) for safety warnings and hazards.

BEFORE STARTING

Considerations Before You Start

- The health of the HEK293T cells is critical for optimal AAV yield.
- Do not overgrow your cells. Pass the cells twice a week during the maintenance phase and do not allow cells to reach 100% confluence (80-90% is ideal).
- Pass and plate the cells the day before the transfection.
- Thaw a new vial of cells after 30 passages.

Procedure

1 Trypsinize and resuspend the HEK293T packaging cells from 2 x T-150 flasks. Cells should be at ~80% confluence. For each T-150 flask:
- Aspirate culture media and rinse once with **10 ml** of PBS.
- Aspirate PBS and add **4 ml** of 0.05% Trypsin/EDTA. Wait ~ **00:02:00** .
- Neutralize trypsin by adding **4 ml** of HI-FBS.
- Pipet back and forth vigorously multiple times to obtain a single cell suspension (no clumps of cells).

2 Pool cells from 2 x T-150 flasks. Adjust volume to **200 ml** with DMEM complete media and mix.

3 Seed all cells in 1 CF2. Return to incubator for **48:00:00** .

4 Trypsinize and resuspend cells from the CF2. Cells should be at ~80% confluence.
- Aspirate culture media and rinse once with 60 mL PBS.
- Aspirate PBS and add **35 ml** of 0.05% Trypsin/EDTA.
- While waiting for the cells to lift-up, aliquot **5 ml** of HI-FBS to a sterile **100 ml** plastic bottle.
- Gently tap the sides of the CF2 to help detach the cells, then transfer the cells into the bottle containing **5 ml** of HI-FBS to neutralize the trypsin.
- Rinse the CF2 with **30 ml** of DMEM complete medium and pool with the cells harvested in the previous step.
- Pipette back and forth vigorously to obtain a single cell suspension (no clumps).
- Add **435 ml** of complete medium (final volume **500 ml**).

5 Seed 250 million cells from the CF2 into 1 CF5. Return to incubator for **24:00:00** - **36:00:00** .

6 Proceed with transfection:
- Calculate the amount of each plasmid needed to have a 1:1:1 molar ratio with **2 mg** total DNA per CF5

Plasmid	Plasmid Size (bp)	DNA Concentration (µg/µl)	Volume of DNA (µl)
RepCap	7,265	1.00	727.6
pHelper	11,854	1.00	1,185
Transfer Plasmid	5,842	1.00	584.2
Total bp	24,961		

- In total we would like 2.5 mg of DNA or 2,500 µg
- Using the total number of base pairs from all three plasmids, we can determine the total µg/bp we need to achieve a 1:1:1 molar ratio of each plasmid:
  2,500 µg/ 24,961 bp = 0.10 µg/bp
- Therefore, for each plasmid we need:

Sample Calculations
RepCap: 0.10 µg/bp × 7,265 bp = 727.6 µg
Volume Needed: 727.6 µg/ 1.0 µg/µl = 727.6 µl
pHelper: 0.10 µg/bp × 11,854 bp = 1185.4 µg
Volume Needed: 1185.4 µg/ 1.0 µg/µl = 1185.4 µl
Transfer Plasmid: 0.10 µg/bp × 5,842 bp = 584.2 µg
Volume Needed: 584.2 µg/ 1.0 µg/µl = 584.2 µl

- Aliquot **176 ml** of OptiMEM into a sterile **250 ml** bottle.
- Aliquot **350 ml** of DMEM + 2% HI-FBS into a sterile **500 ml** bottle.
- Add the plasmid DNA into the bottle containing the Opti-MEM. Mix well.
- Add **7.5 ml** of PEI (1:3 µg DNA to µg PEI ratio). Shake the bottle up/down vigorously for **00:00:30** (it's okay to make bubbles).
- Incubate at room temperature for **00:15:00** . Note that longer incubation times can reduce transfection efficiency.
- Add the OptiMEM + DNA + PEI solution to the bottle containing **350 ml** of DMEM + 2% HI-FBS. Mix well.
- Take the CF5 out of the incubator and pour the media into a waste container.
- Carefully add the OptiMEM + DNA + PEI solution to the CF5. Make sure that all five layers are covered with media. 293T cells are delicate and detach very easily - media should always be added away from the cells (not poured on them) and can be adjusted by carefully tilting the CF5 back and forth.

Pro-Tip
To help distribute the media amongst the five layers, tilt the CF5 such that the media goes toward the cap. If the media touches the cap, replace it with a new one before putting the CF5 in the incubator.

- Return the CF5 to the incubator for **96:00:00** . This incubation time can be adjusted depending upon the serotype. Often AAV2 are harvested at **48:00:00** - **72:00:00** , while other serotypes are harvested at **96:00:00** - **120:00:00** .

7 Harvest cells and media by tapping the sides of the CF5 on a soft surface. Cells should detach easily.

8 Transfer cells and media into **50 ml** conical tubes (or **500 ml** conical vessels).

9 Rinse CF5 once with **20 ml** of PBS and add to a new **50 ml** conical tube.

10 Centrifuge at 3,000 g or max speed on a tabletop centrifuge for **00:15:00** at **4 °C** to pellet the cells.

11 Keep the cell pellet on ice and transfer supernatant to a sterile 500 mL bottle.

12 Process the supernatant as follows:

- Filter through a 0.45 µm PES membrane.
- Add **25 ml** of PEG solution to each **100 ml** of supernatant. Split into 2 x **500 ml** sterile bottles as needed.
- Add stir bar and stir slowly at **4 °C** for **01:00:00** , then keep at **4 °C** for **03:00:00** without stirring to allow full precipitation. Precipitation of the viruses can proceed overnight at **4 °C** if needed.
- Transfer the entire sample to **50 ml** conical tubes and centrifuge at 2,818 g for **00:15:00** at **4 °C** .
- Discard the supernatant and resuspend the virus (small pellet) in **10 ml** PBS + 0.001% pluronic F68 + 200mM NaCl. Pipet back and forth to resuspend the virus completely.
- Keep resuspended pellet on ice.

13 Process the cell pellet as follows:

- Resuspend and lyse the cells by adding **10 ml** of PBS + 0.001% pluronic F68 + 200mM NaCl and sonicating 4 x 1 sec pulses with at least **00:15:00** on ice between each pulse, 50% amplitude. Return to ice between each sonication to avoid overheating of the sample.
- Pellet cell debris by centrifugation at 3,220 g for **00:15:00** at **4 °C** .
- Transfer the cleared lysate to the tube containing the resuspended virus from step 12 above.

14 Add 50 units of benzonase per mL of viral suspension in PBS (you should have ~ **20 ml** from steps 12 & 13). Benzonase is an endonuclease that will degrade any residual DNA carried over from the packaging process.

15 Incubate at **37 °C** for **00:45:00** .

16 Transfer the viral suspension to centrifuge tubes and centrifuge at **4 °C** at **2415 x g** for **00:10:00** .

17 Transfer the clarified supernatant to a new tube and proceed with purification.

Note: an aliquot of the solution can be kept for qPCR to determine the vector loss due to the purification. Please note that reagents presents in the media may impair PCR efficiency.

18 You are now ready to purify your prep. The clarified supernatant can be kept overnight at 4 °C before proceeding with the purification protocol.