

SOP: Viral Gene Delivery and Evaluation Using Flow Cytometry

Approvals:

Preparer: Dr. Dominique Ingato
Reviewer: Dr. Barbara Juncosa

Date: 29AUG21
Date: 31AUG21

1. Purpose:

- 1.1. To transduce mammalian cells with the gene for green fluorescence protein (GFP) using adeno-associated virus (AAV); to evaluate transduction efficiency using flow cytometry.

2. Scope:

- 2.1. This SOP applies to transduction of Chinese hamster ovary (CHO) cells using AAV GFP. This method employs a Luminex Guava easyCyte Flow Cytometer controlled by guavaSoft Software.

3. Responsibilities:

- 3.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
- 3.2. It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

4. References:

- 4.1. Vector Biolabs User Manual for Ready-to-use AAV Products (rev. 20APR16)
- 4.2. Invitrogen Countess II FL automated cell counter User Guide (rev. 2019)
- 4.3. Luminex Guava easyCyte HT System User Guide (rev. 04/2020)

5. Precautions:

- 5.1. Use BSL1 safety measures and discard waste in biohazard containers.

6. Materials:

- 6.1. Biological safety cabinet
- 6.2. CO₂ incubator
- 6.3. Microcentrifuge
- 6.4. Life Technologies Countess II automated cell counter
- 6.5. Luminex Guava easyCyte HT flow cytometer
- 6.6. Vials of CHO 1-15 cells (ATCC CRL-9606)
- 6.7. F-12 (Ham) Medium, ThermoFisher # 11765-047
- 6.8. Fetal bovine serum
- 6.9. Gentamicin (50 mg/mL), ThermoFisher # 15750-060
- 6.10. Trypsin/EDTA solution (0.25%)
- 6.11. AAV-CMV-GFP (AAV-DJ) AAV, Vector Biolabs # 7101
- 6.12. 1X PBS
- 6.13. P20, P200, and P1000 micropipettes and compatible tips
- 6.14. 1.5 mL microfuge tubes and holder

SOP: Viral Gene Delivery and Evaluation Using Flow Cytometry

- 6.15. T-25 vented tissue culture flasks
- 6.16. 0.4% trypan blue stain
- 6.17. Guava easyCheck Kit
- 6.18. Bleach
- 6.19. DI water
- 6.20. Guava Instrument Cleaning Fluid (ICF)
- 6.21. 96-well plate (round bottom)

7. Procedure:

7.1. Preparation for transduction

- 7.1.1. Prepare complete media
 - 7.1.1.1. In the biosafety cabinet, aseptically add 100 mL of FBS and 1 mL of Gentamicin to 1 L of F-12 (Ham) media.
 - 7.1.1.2. Label and store complete media at 2-8°C.
- 7.1.2. Prepare AAV
 - 7.1.2.1. Dilute AAV 1:100 in 1X PBS.
 - 7.1.2.2. Store at 4°C for 2-3 weeks.
- 7.1.3. Prepare CHO cell plates
 - 7.1.3.1. Plate 1×10^6 cells/well in 2 mL media/well in a 6-well plate.
 - 7.1.3.2. Incubate the plate in the CO₂ incubator for 24 hours.

7.2. Transduction and preparation for assessment

- 7.2.1. Remove media from the 6-well plate.
- 7.2.2. Add the following to each of the 6 wells: 1 mL media; 1 mL media with 10 µL diluted AAV; 1 mL media with 20 µL diluted AAV; 1 mL media with 30 µL diluted AAV; 1 mL media with 40 µL diluted AAV; and 1 mL media with 50 µL diluted AAV.
- 7.2.3. Incubate the plate in the CO₂ incubator for 48 hours.
- 7.2.4. Remove and discard the media
- 7.2.5. Add 1 mL of 0.25% trypsin/EDTA solution and incubate the plate in the CO₂ incubator for 5 minutes.
- 7.2.6. Gently pipette in each well and transfer the contents of each well to a 1.5 mL microcentrifuge tube.
- 7.2.7. Centrifuge at 1200 rpm for 5 minutes.
- 7.2.8. Remove the supernatant, leaving the pellet at the bottom of the tube.
- 7.2.9. Resuspend the pellet in 1 mL of 1x PBS.

7.3. Assessment of viability post-transduction

- 7.3.1. Turn on the Countess II automated cell counter by pressing the switch on the lower right-hand corner of the back of the instrument.
- 7.3.2. Prepare samples by adding 10 µL of sample to 10 µL of 0.4% trypan blue stain.
- 7.3.3. Open Countess cell counting chamber slide and load 12 µL of sample mixture into Side A.
- 7.3.4. Insert the slide (Side A first) into the slide port until it clicks.
- 7.3.5. Set exposure to “light source” and press “capture” on the touchscreen.
- 7.3.6. Record your data.

SOP: Viral Gene Delivery and Evaluation Using Flow Cytometry

7.3.7. Remove the slide and dispose of it in the biohazard container.

7.3.8. Turn off the Countess II automated cell counter by pressing the switch on the lower right-hand corner of the back of the instrument.

7.4. Assessment of transduction efficiency

7.4.1. Perform instrument cleaning

7.4.1.1. Turn on the Luminex Guava easyCyte HT flow cytometer by pressing the switch on the lower right-hand corner of the back of the instrument.

7.4.1.2. Turn on the laptop computer.

7.4.1.3. Open the guavaSoft software by clicking the icon on the desktop.

7.4.1.4. From the main menu, click Cleaning.

7.4.1.5. Click Start Clean, and the plate will be ejected.

7.4.1.6. Load the following tubes in the instrument:

- Load seven tubes filled with DI water in positions w1, w2, w3, w4, w5, w6, and tube position 1.
- Load a tube containing 100 μ L of 100% bleach in position 2.
- Load two tubes with a mixture of 10% bleach and Guava ICF in tube positions 3 and 4.
- Load a tube filled with DI water in any tube position (5-9) for the capillary shutdown. Then select this position on the plate map.

7.4.1.7. Click OK, and the instrument will run the cleaning protocol.

7.4.2. Perform Guava easyCheck.

7.4.2.1. Prepare a 1:20 dilution of the Guava easyCheck bead reagent by adding 380 μ L of Guava Check Diluent to 20 μ L of easyCheck bead reagent. Mix thoroughly.

7.4.2.2. From the main menu, click easyCheck.

7.4.2.3. Enter the following in the appropriate fields

- The easyCheck bead lot #, which can be found on the easyCheck card.
- The bead expiration date, which can be found on the easyCheck beads reagent box.
- The expected particles/mL, which can be found on the easyCheck card.

7.4.2.4. Click Start, and the sample tray will open.

7.4.2.5. Load the following tubes in the instrument:

- Load seven tubes filled with DI water in positions w2, w4 and w5.
- Load empty tubes in w3 and w6.
- Load a tube containing 100 μ L of 100% bleach in w1.
- Load the easyCheck sample in any well of the microplate. Click to select the well with beads.

7.4.2.6. Click OK, and the instrument will run the easyCheck protocol.

7.4.2.7. If any result for particles/mL falls outside of $\pm 10\%$ of the expected value, the result is outside of the acceptable range. In this case, notify the instructor.

7.4.3. Assess transduction efficiency

7.4.3.1. Add 100 μ L of resuspended cell samples (from 7.2.9) to the first 6 wells of a round-bottom 96-well plate.

SOP: Viral Gene Delivery and Evaluation Using Flow Cytometry

- 7.4.3.2. From the guavaSoft main menu, click InCyte. The screen will now show multiple plots.
- 7.4.3.3. Change the plot on the right to a histogram by clicking the Plot type icon and selecting Histogram.
- 7.4.3.4. Click on the x-axis label and select Green-B Fluorescence (GRN-B-HLog).
- 7.4.3.5. Click the Edit Worklist button. Define the worklist parameters by selecting the wells on the screen that contain samples.
- 7.4.3.6. Click Run Worklist, and the tray will eject.
- 7.4.3.7. Place the plate on the tray with well A1 in the top-right corner.
- 7.4.3.8. Load the following tubes in the instrument:
 - Load seven tubes filled with DI water in positions w2, w4 and w5.
 - Load empty tubes in w3 and w6.
 - Load a tube containing 100 μ L of 100% bleach in w1.
 - Load a tube containing ICF in position 10.
 - Load a tube for parking the capillary.
- 7.4.3.9. Click OK to load the sample tray.
- 7.4.3.10. The system will prompt you to adjust settings. Select your control well (that containing cells that have not been treated with AAV), then click OK.
- 7.4.3.11. Go to the Tools menu and click Gain Controls. Adjust the GRN-B Gain so that you see a smooth curve on the histogram while your control sample is being analyzed.
- 7.4.3.12. When you are finished, click the Next Step button.
- 7.4.3.13. Click Resume Worklist to analyze all samples.
- 7.4.4. Analyze your data
 - 7.4.4.1. After the system finishes running the worklist, a copy of your data will be automatically loaded into the analysis control window.
 - 7.4.4.2. On the left-hand side plot (forward vs side scatter), create an elliptical region around the concentrated area of cells by clicking the New Region icon, selecting Elliptical region, and drawing the region around the cells. The system will prompt you to name the region. Name it as R1.
 - 7.4.4.3. Create a Gate based on R1. Click the Elliptical region in the left-hand side plot and drag it to the right-hand side histogram.
 - 7.4.4.4. Create a Stat marker. In the histogram, click the New Stat Marker icon. Select Percent and label it as “Non-fluorescent cells.” Draw the marker so that it aligns with the region defined by the control histogram.
 - 7.4.4.5. Create another Stat marker. In the histogram, click the New Stat Marker icon. Select Percent and label it as “Fluorescent cells.” Draw the marker so that it aligns with the region to the right of the first stat marker.
 - 7.4.4.6. To view current run stats, click Tools then Show Current Run Stats. You will be able to see the percentage of “Fluorescent cells” in each well.
 - 7.4.4.7. Record your data.
- 7.4.5. Perform instrument cleaning
 - 7.4.5.1. Follow protocol described in 7.4.1

SOP: Viral Gene Delivery and Evaluation Using Flow Cytometry

7.4.5.2. Perform the Shut Down the Capillary procedure to shut down the capillary with a tube of DI water to keep it moist while the instrument is not in use.

7.4.5.3. Exit the software and turn off the laptop.

7.4.5.4. Turn off the Luminex Guava easyCyte HT flow cytometer by pressing the switch on the lower right-hand corner of the back of the instrument.

8. History:

Name	Date	Amendment
Dr. Dominique Ingato	31AUG21	Initial release