

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 1 of 12</b>

## 1.0 PURPOSE

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

## 2.0 SCOPE

- 2.1 This SOP applies to transduction of Human Embryonic Kidney (HEK) 293 cells using AAV GFP. Evaluation will be conducted on the BD Accuri C6 Plus System.

Software.

## 3.0 REFERENCES

- 3.1 This Lab was adapted from Dr. Dominique Ingato's SOP regarding Viral Gene Delivery and Evaluation using Flow Cytometry

Document #/Reference	Title
1 / Dr. Dominique Ingato . MiraCosta College	SOP: Viral gene delivery and evaluation using flow cytometry
BD 23-18013-00	BD Accuri C6 Plus System Users Guide
BIOT222-01	SOP - Introduction, Maintenance, and Operation of the BD Accuri C6 Plus Cytometer

## 4.0 DEFINITIONS

Terms	Definitions
Adeno-associated Virus (AAV)	A small class of viruses that can infect humans. Here, the virus serves as a vector and has lost its innate viral package.
Green Fluorescent Protein (GFP)	A protein that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. A common fluorophore used in flow cytometry.
Fluorophore	A molecule with fluorescent properties.
Gating Strategy	In data analysis, the combination of scaffolding graphs to identify subsets of cells.
Forward Scatter (FSc)	A flow cytometer parameter to measure the size of cells.
Side Scatter (SSc)	A flow cytometer parameter to measure internal complexity of cells.

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 2 of 12</b>

## 5.0 SAFETY

- 5.1 Use of BSL-1 Safety Measures and discard all waste in biohazard.
- 5.2 Users should always wear goggles, lab coats, and gloves when handling samples and the cytometer.
- 5.3 Operators must be aware of safety symbols located on and around the instrument, indicating specific hazards including:
- Biological Hazard
  - Electrical Hazard
  - Laser Hazard

## 6.0 RESPONSIBILITIES

Role	Responsibilities
Instructors / Lab Technicians	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.
Students/Trainees	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.

## 7.0 MATERIALS

- HEK293 cells
- Phosphate Buffered Saline (Gibco / 10010)
- BD CS&T RUO beads ( BD Biosciences / 661414))
- BD FACSClean (BD Biosciences / 340345)

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 3 of 12</b>

- DI Water
- 12 x 75-mm capped polystyrene tube (Falcon/Corning / 352058 or 352054)
- Phosphate Buffered Saline
- AAV/DJ-CMV-eGFP (Vector Biolabs / 7101)
- BD Pharmingen Stain Buffer (BD Biosciences / 554656) or PBS + 1% FBS
- F-12 Media (Gibco / 11765054)
- HI Fetal Bovine Serum (Gibco / 61640071)

## **8.0 EQUIPMENT**

- Biological Safety Cabinet
- BD Accuri C6 Plus System
- Centrifuge
- 2-8C Refrigerator
- 37C, 5% CO<sub>2</sub>, 70-80% Humidity incubator
- -80C Freezer (For storage of Viral Vector)

## **9.0 PROCEDURE**

### **9.1 Transduction Preparation (Day 0)**

9.1.1 Retrieve prepared Complete Media (F12 + 10% FBS)

9.1.2 Prepare AAV

9.1.2.1 Prepare 200 $\mu$ L of 1:100 diluted AAV in 1X PBS and store at 2-8C.

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 4 of 12</b>

9.1.2.2 Retrieve a prepared 6-well plate of HEK293 from the incubator

9.2 Transduction of HEK293 Cells (Day 0)

9.2.1 Prepare Cells

9.2.1.1 Remove the HEK293 seeded 6-well plate from incubator

(prepared by instructor), being sure not to disrupt the monolayer.

(The cells will be adherent and should not come off when gently removing media).

9.2.1.2 Remove old media and add the following components to each

well:

9.2.1.3 Well 1 – 4mL of Media

9.2.1.4 Well 2 – 4mL of media with 10µL of diluted AAV

9.2.1.5 Well 3 – 4mL of media with 20µL of diluted AAV

9.2.1.6 Well 4 – 4mL of media with 30µL of diluted AAV

9.2.1.7 Well 5 – 4mL of media with 40µL of diluted AAV

9.2.1.8 Well 6 – 4mL of media with 50µL of diluted AAV

9.2.2 Agitate the plate gently forward/backward and side/side to accurately disperse media and AAV. DO NOT agitate in a circular fashion, as the materials will collect in the center.

9.2.3 Incubate plate at 37C for 48 hours

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 5 of 12</b>

9.3 Cytometer Setup and Calibration (Day 2)

9.3.1 Before starting the cytometer, check the fluid levels in all of the bottles and fill/empty accordingly:

9.3.1.1 Check the Sheath Fluid Bottle (DI H<sub>2</sub>O)

9.3.1.2 Check the BD FACSClean and detergent solution bottles

9.3.1.3 Check the waste bottle to ensure adequate capacity.

9.4 Starting up the cytometer:

9.4.1 Open the BD Accuri C6 Plus Program on the computer

9.4.2 Prepare a FACS tube containing 2mL or more of DI water.

9.4.3 Push the sample stage back, place the tube of water over the SIP, and while holding the tube, pull the sample stage forward to support the tube. There may already be one on, but replace with a new fresh tube.

9.4.4 Start the instrument by pressing the Power Icon (⏻) on the front of the machine. The power indicator will flash blue and the traffic light will show as yellow.

9.4.5 Allow 15 minutes for the Cytometer Lasers to warm up. Record when the instrument was turned on, and when it has finished preparing.

9.4.6 Once the cytometer is ready to go, the traffic light will turn from yellow to green and the power indicator will no longer flash

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 6 of 12</b>

9.5 Preparing for Instrument QC:

9.5.1 Label a 12 × 75-mm capped polystyrene tube (FACS Tube) as “CS&T.”

9.5.2 Add 500µL of PBS to the tube as diluent to the tube.

9.5.3 Thoroughly mix the BD CS&T RUO beads vial. Invert the vial 10 times, or vortex the vial at medium speed for 5–10 seconds.

9.5.4 Add 1 drop of the beads to the tube. Avoid dripping the beads down the side of the tube when diluting them. This can lead to low bead counts during acquisition.

9.5.4.1 If you will not be using the diluted beads right away, store the diluted beads at 2°C–8°C in the dark. Protect the diluted beads from light.

9.5.5 Vortex the tube immediately before acquiring the sample

9.6 Running Instrument QC:

9.6.1 Click Instrument QC in the upper-right corner of the workspace.

9.6.2 After the module opens, select the bead lot from the BD CS&T Bead Lot menu. The last bead lot run appears as the default.

9.6.2.1 If using a new lot of beads, you need to install the corresponding bead lot file. Check Lot Number on the bead vial.

9.6.3 Enter your Operator ID and the Lab Director ID.

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 7 of 12</b>

9.6.4 Run the CS&T beads. Mix the tube before loading, then place it on the SIP. Select the RUN button.

9.6.5 Wait for acquisition to complete. Watch the calibration to ensure no issues while running.

9.6.6 Once complete, remove the tube and rinse the SIP by returning the tube of DI water to the SIP.

9.6.7 Adjust the regions of the dot plots if necessary. You are now ready to acquire samples.

#### 9.7 Preparation of Transduced Samples

9.7.1 Warm media in a 37C water bath.

9.7.2 Label 6 microcentrifuge tubes and 6 FACS tubes as Well 1 through 6.

9.7.3 Remove and discard the media.

9.7.4 Wash wells with 1mL of 1X PBS.

9.7.5 Add 0.5mL of dissociation reagent (0.25% Trypsin/EDTA) to each well and incubate at 37C for 2-5 minutes, or until cells begin to detach from the wells.

9.7.6 Add 2x amount of warm complete media to neutralize the dissociation reagent and transfer cell solutions to the labeled microcentrifuge tube.

Spin down at 400 x g for 5 minutes and remove supernatant.

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 8 of 12</b>

9.7.7 Resuspend the pellet in 1mL of PBS. Prepare the cells as a 1:2 dilution in 50µL and count using the automated cell counter. Record the viability and total viable cells for each sample.

9.7.8 Transfer cell suspensions from microcentrifuge tube to FACS tubes. Cover and store at 2-8C until ready to acquire on the cytometer.

9.8 Set the Acquisition Parameters on the Cytometer

9.8.1 Upon opening the program, a new workspace is automatically generated. If you are not the first operator to read your sample, ensure that the previous workspace has been saved, then you select File > New Workspace to create a new one.

9.8.2 Set a Run Limit of 50,000 events. This indicates when the collection will end. This is completed by selecting **Run with Limits**, selecting the checkbox next to the events field, and typing in the number of events. You can choose to focus on a gated population or all cells.

9.8.3 Set a Fluidics Rate of Medium. This indicates how fast to collect the sample. Depending on the cell concentration and the size of the cells, this can be adjusted accordingly. A recommended flow rate is between 1,000 to 2,500 events per second.

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 9 of 12</b>

9.8.4 Set a Threshold of 50,000 for FSC-H. The threshold sets the cut off for eliminating debris and noise from cell samples. By default, it is set to 80,000, but we will set it slightly lower.

#### 9.9 Creating Sample Gating Strategy on the Cytometer

9.9.1 Confirm that the first graph displayed states “FSC-A” on the X-Axis for Forward Scatter Area, and “SSC-A” on the Y-Axis for Side Scatter Area. Rename plot to “Cells.”

9.9.2 Select the oval gate icon  and add an oval gate to the bottom right of the graph.

9.9.3 Select the Dot Plot Icon  to add a new graph. To change the specifications, select the **Plot Spec** icon in the bottom right of the graph. Set “FSC-A” on the X-Axis for Forward Scatter Area, and “FSC-H” on the Y-Axis for Forward Scatter Height.

9.9.4 Rename the Plot to “Singlets.” Select the **Gate** icon at the top left. Select **Include** and select the gate from the Cells graph.

9.9.5 Select the Polygon gate icon.  Draw a diagonal, rectangular 4-point box. This will be used to separate single cells from clumps of cells that may register incorrectly.

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 10 of 12</b>

9.9.6 Select the Dot Plot Icon to add a new graph. To change the specifications, select the **Plot Spec** icon in the bottom right of the graph. Set “FITC” on the X-Axis for GFP expression and “SSC-A” on the Y-Axis for Side Scatter Area. If not already, change the X-Axis to “Linear” instead of Log.

9.9.7 Rename the Plot to “GFP+.” Select the **Gate** icon at the top left. Select **Include** and select the gate from the Singlets graph.

9.9.8 Select the Box gate icon.  Draw a box in the right half of the graph.

#### 9.10 Sample Acquisition

9.10.1 Select an empty sample well and enter the first sample’s name “Well 1\_Initials” in the text box above the 96-well grid. You can name samples at any point, but it is highly suggested you record the name before as to not forget.

9.10.2 Resuspend your cells in the sample tube and place tube on the SIP.

9.10.3 Click **Run** to start the sample collection. Save your workspace. Adjust all gates as needed.

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 11 of 12</b>

9.10.4 The selected Well will flash blue during acquisition. Once the 100,000 events are recorded, the run will finish and the selected Well will stop flashing. Remove the tube from the SIP

9.10.5 Select the next empty sample well and enter the next sample's name "Well 2\_Initials" in the text box above the 96-well grid.

9.10.6 Resuspend your cells in the sample tube and place tube on the SIP.

9.10.7 Click **Run** to start the sample collection.

9.10.8 Repeat the process for all remaining samples

#### 9.11 Data Analysis

9.11.1 Select the Batch Analysis tab to compare the GFP expression from every sample.

9.11.2 Click the **Batch Analysis** tab. Select the **Show Plots from Collect** checkbox.

9.11.3 Select all 3 gates by marking the checkbox under that plot.

9.11.4 Select the samples to analyze (all).

9.11.5 Select the **Show Statistics with All Plots** check box to show statistics.

9.11.6 Further Analyses can be completed in a software analysis package such as FlowJo or FCS Express.

#### 9.12 Export the Data.

	<b>STANDARD OPERATING PROCEDURE</b>	
	<b>Viral Gene Delivery and Evaluation by Flow Cytometry</b>	
<b>Document Number: SOP-BIOT222-02</b>	<b>Version: 01</b>	<b>Page 12 of 12</b>

9.12.1 Select **Export to PowerPoint** and include statistics.

9.12.2 Navigate to the destination folder, record the file name and document the full file pathway.

9.12.3 Print out the PowerPoint and attach to this Lab Workbook.

## 10.0 ATTACHMENTS

- SOP – Viral Gene Delivery and Evaluation using Flow Cytometry
  - Document Number 1, 31Aug2023, Dr. Dominique Ingato