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Protocol: Cas9 detection using flow cytometry

Adapted from:
Abcam Direct flow cytometry protocol
Abcam Cell fixation and permeabilization kit protocol

This is not an activity assay. This assay quantifies Cas9 expression, not activity. There are instances in which Cas9 expression and activity do not correlate. To quantify Cas9 activity, please refer to “Protocol: Assaying Cas9 activity with an EGFP reporter”.

This assay uses flow cytometry to quantify Cas9 expression on an individual cell basis. It is potentially useful for visualizing Cas9 expression in constructs that may be difficult to express, quantifying Cas9 expression over the course of a screen, and observing induction with inducible vectors. Cell number is not of great importance (any quantity from 5.0E5 to 4.0E6 cells will suffice).

Materials and reagents required:

- Parental (no Cas9) cells
- Cas9 expressing cells
- Alexa Fluor 647 anti-Cas9 antibody (Cell Signaling Technology, #48796)
- Fixation and permeabilization kit (Abcam, #185917)
- Flow buffer (PBS, 2% FBS, 5μM EDTA)
- Chilled PBS
- 96-well V-bottom plate (Corning #3357)
- Centrifuge
- Flow cytometer (ex. Accuri)

To stain/flow:

1. Trypsinize and count cells.

2. Determine the number of wells needed. At a minimum, include 2 wells of parental cells (1 stained and 1 unstained) and 2 wells of Cas9+ cells (1 stained and 1

unstained). For each well, spin down 1E6 cells and resuspend in 50 uL chilled PBS.

3. Add 50uL cell mixture (cells in PBS) per well to a 96-well V-bottom plate, including:
    a. 2 NIC (non-infection control) wells - 1 stained and 1 unstained control
    b. 1 well Cas9+ cell line stained with antibody
    c. 1 well unstained Cas9+ cells

4. Turn off hood light (light should remain off until protocol is complete). To fix the cells, add 100uL Reagent A (Fixation Medium) from the Abcam Cell Fixation & Permeabilization Kit. Mix thoroughly.

5. Cover the plate with foil. Incubate for 15 mins at room temp.

6. Add 100 uL of chilled PBS, pipette up and down thoroughly, and centrifuge cells for 5 minutes at 300 x g.

7. Remove supernatant with aspirator. To permeabilize the cells, add 100uL Reagent B (Permeabilization Medium) from the Abcam Cell Fixation & Permeabilization Kit. To stain cells, add 2 uL of antibody. Pipette up and down thoroughly.

8. Incubate for 30 mins at room temp in the dark (lights off, covered in foil).

9. Add 200uL chilled PBS, resuspend by pipetting up and down, and centrifuge for 5 mins at 300 x g to wash.

10. Aspirate PBS, taking care not to disturb the cell pellets. Wash 2x more (for a total of 3 washes, spinning for 5 mins at 300 x g each time).

11. Aspirate PBS and resuspend with 200uL chilled flow buffer.

12. Read out on FL- 4 of the Accuri flow cytometer.
    a. Gate live cells using FSC/SSC
    b. Use the NIC unstained control well to set gates on FL-4

*In an initial optimization experiment all combinations of 5.5e5, 1e6, 2e6, 4e6 cells/well, and antibody volumes of 1, 2, 3, 4 and 5uL were tested. Neither cell number nor antibody volume seemed to have a large effect on readout. As a result, any of these cell numbers with any antibody volume should work. In follow up experiments, 1e6 with 2uL was used.