Flow Cytometry

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DOI:

dx.doi.org/10.17504/protocols.io.whjfb4n

Protocol Citation: Thomas P Howard, Taylor Arnoff . Flow Cytometry.

protocols.io

<https://dx.doi.org/10.17504/protocols.io.whjfb4n>

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Created: Dec 18, 2018

Last Modified: Dec 27, 2018

PROTOCOL integer ID:
18699

Plate cells and perform drug treatments

- 1 Cells were plated at 2 million cells per 10 cm plate and allowed to adhere prior to drug addition. Independent plates were used for propidium iodide (PI) and Annexin V stains.
- 2 For SMARCB1 inducible cells, doxycycline was added at a final concentration of 1 µg/mL (from a 5 mg/mL stock, Clonetechn # NC0424034). Cells were allowed to grow for 48 hours.
- 3 Cells were treated with either DMSO (0.05% final), idasanutlin (MedChemExpress, Monmouth Junction, NJ # HY-15676, 10 mM stock) (1 µM final), or ATSP-7041 (5 µM final) for 24 hours.

Flow cytometry general preparation

- 4 Media was aspirated off from cells to be analyzed with PI stain. For cells to be analyzed with Annexin V stain, the media was saved to capture any floating cells.
- 5 Cells were trypsinized by adding 2 mL of Trypsin-EDTA 0.25% (Thermo Fisher Scientific # 25200114) per 10 cm plate. Cells were allowed to trypsinize for ~1-2 minutes at 37°C.

Note: Keep trypsinization time as short as possible.
- 6 Cells to be analyzed with PI stain were quenched with 8 mL of fresh media. Cells to be analyzed with Annexin V stain were quenched with the media saved from step 4.
- 7 Cells were spun down at 1000 rpm for 5 minutes at room temperature.
- 8 Cells were washed once with 10 mL of cold PBS and counted using a Beckman Coulter Counter (Beckman Coulter Life Sciences, Z Series Coulter Counter # 6605698).
- 9 Cells were spun down at 1000 rpm for 5 minutes at 4°C.
- 10 Cells were resuspended at 2 million cells/mL in cold PBS.
- 11 1 mL of cells was transferred into a 1.7 mL microcentrifuge tube (2 million cells/tube).

- 12** Cells were spun down at 500 x g for 5 minutes at 4°C. Proceed to step 13 for Annexin V staining protocol. Proceed to step 21 for PI staining protocol.

Annexin V Staining Protocol

- 13** Cells were resuspended in 0.5 mL of 1X Binding Buffer from the BD Annexin V: FITC Apoptosis Detection Kit I (Thermo Fisher Scientific # BDB556547) diluted from 10X stock with dH₂O at a concentration of 4 million cells/mL.
- 14** 100 µL of solution (4×10^5 cells) was transferred into a flow cytometry tube through a filter cap (Thermo Fisher Scientific # 352235).
- 15** 5 µL of PI and 5 µL of Annexin V from the BD Annexin V: FITC Apoptosis Detection Kit I were added per tube.
- 16** For one control (DMSO) condition for each cell line the following was performed:
- 100 µL of stock cell solution had nothing added.
 - 100 µL of stock cell solution had 5 µL of only Annexin V added.
 - 100 µL of stock cell solution had 5 µL of only PI added.
- 17** Cells were vortexed gently and incubated for 15 minutes in the dark at room temperature.
- 18** 400 µL of 1X Binding Buffer was added to each tube. Samples were analyzed by flow cytometry within 1 hour.

Annexin V Analysis

- 19** Cells were run on an SA3800 Spectral Analyzer (Sony Biotechnology, San Jose, CA) capturing at least 20,000 events.

Using the unstained and single stained control samples, spectral references for PI and FITC in

- 20 these cells were calculated and used to unmix the two signals for the dual stained samples. Unmixed data were exported as .fcs files and analyzed in FlowJo 10. Quadrants were chosen based on the unstained and single stained control samples and applied consistently to all samples from a cell type.

PI Staining Protocol

- 21 Cells were resuspended in 100 μ L of 1X PBS.
- 22 900 μ L of fresh 80% ethanol was added to fix the cells (final concentration is ~70% ethanol).
- 23 Cells were incubated on ice for 2 hours.
- 24 Cells were spun down at 500 x g for 5 minutes at 4°C.
- 25 A pipette was used to gently remove the 70% ethanol.
- 26 500 μ L of the FxCycle PI/RNASE (Thermo Fisher Scientific # F10797) was added per sample, and samples were incubated in the dark for 15 minutes at room temperature.

PI Analysis

- 27 Cells were run on an SA3800 Spectral Analyzer capturing at least 20,000 events. Data were exported as .fcs files.
- 28 PI histogram distributions were analyzed using ModFit LT Version 5. In the DMSO treated cells,

singletons were selected from a plot of PI-height vs PI-area. In the DMSO treated cells, the ranges of diploid 2N and 4N cells were selected, and then the best fit unbiased model was calculated. The same model was applied to samples of all other conditions within the same cell type.

- 29** The difference in percentage of G₀/G₁ cells between compound and DMSO treated samples was computed, and three biological replicates were averaged.