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Staining for FACS Sorting of Live Cells

In 1 collection

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol describes Hoechst 33342 staining and FACS sorting of Hap-1 cells in preparation for Saturation Genome Editing experiments.

Materials

Required reagents
Hoechst 33342, 12.3 mg/ml in aqueous solution (20mM); ThermoScientific # 62249
PBS, 1x
IMDM+10%FBS
FBS (heat inactivated, 1mL)
Trypsin
Trypan blue

Required plasticware
Cell counter
50mL Falcon tubes, 2 sets, for collection and preparation of conditioned media
50mL Falcon tube for harvested cells
15mL Falcon tubes, 2 sets, to carry cells before and after sorting
50mL conical tube with filter
Cell strainer

Before start

Prepare a 1mg/mL dilution of Hoechst 33342 (12.3 mg/ml) in water. Determine the amount of dye needed based on the number of culture dishes that need staining and harvesting. Hoechst 33342 precipitates in PBS; only use ddH2O for dilutions. Keep dye protected from light.

Recommended Hoechst 33342 (HO) staining concentration for live cells is 1-5ug/mL. To determine the optimal concentration of Hoechst 33342 for live staining of HAP1 cells, we ran a calibration experiment using 2, 4, 8ug/mL live staining and obtained optimal results with 2ug/mL as well as 4ug/mL Hoechst 33342. We chose to use 2ug/mL Hoechst 33342 for a sorting experiment.

Preparing Staining Media

1h

1 To perform staining in vitro, both media and trypsin should contain the same HO concentration. Prepare enough IMDM+10%FBS+0.2%HO and Trypsin+0.2%HO for the number of culture plates that need staining. 1h

For each 15cm plate, prepare the following:
- 30mL IMDM+10%FBS+0.2%HO (20mL for staining incubation at 37C, 10mL to neutralize trypsin)
- 2mL Trypsin+0.2%HO
- 20mL PBS+1%FBS (assuming 100×10^6 cells in a 15mL plate and resuspending cells at 5×10^6 cells/mL for sorting)

**Prepare 50% conditioned media. At least 01:00:00 before the scheduled sorting time, label 50mL Falcon tubes for conditioned media. Transfer culture media (instead of aspirating) from cell plates to a 50mL Falcon tube, centrifuge at 1000rpm for 5-10minutes, collect supernatant and filter in a fresh 50mL tube. Add same volume of fresh IMDM+10%FBS to obtain 50% conditioned media.

Staining and harvesting cells

45m

2 Collect and transfer media from culture plate to a 50mL Falcon tube and prepare conditioned media.

3 Wash culture plate with 10mL PBS once. 45m
Add 20mL IMDM+10%FBS+0.2%HO media and incubate at 37°C for 00:45:00 (live staining).
Set up trypan blue and cell counter.

4 Aspirate media and wash once with PBS. Add 2mL Trypsin + 0.2%HO and incubate at 37°C for 4 minutes. Make sure cells are detaching from plate by hitting gently on side of hood. Neutralize trypsin by adding 10mL IMDM+10%FBS+0.2%HO to the cells, gently resuspend and collect cells in a 50mL Falcon tube.

5 Resuspend cells homogeneously. Count cells and determine resuspension volume to a concentration of 5×10^6 cell/mL.
Centrifuge cells at 500rpm for 5-10 minutes. Discard supernatant and resuspend in PBS+1% heat-inactivated FBS (Or equivalent sorting buffer) to 5×10^6 cell/mL.

6 If sorting 100×10^6 cells, resuspend cell pellet in 20mL sort buffer. Using a cell strainer to break cell clumps, aliquot 5mL cells each in 15mL Falcon tubes.

Label 15mL Falcon tubes for collection of sorted cells. Add 1mL conditioned media to each tube and seal. Keep sterile.

7 Prepare a clean storage box to transport cells to FACS machine. Bring clean and labelled 15mL Falcon tubes (with 1mL conditioned media) for cell collection, gloves, notebook.

FACS

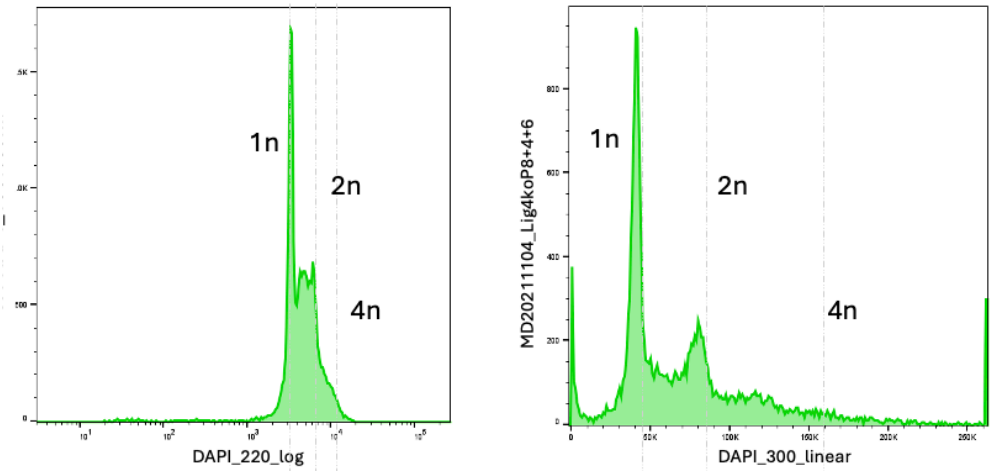
2w

8 Calibrate FSC and SSC according to your machine's settings.

Establishing gating with an unstained sample is recommended.

Most FACS machines DAPI laser is sufficient for Hoechst 33342 stain.

Final gate should be drawn around 1n peak to sort for halpoid cells. A well-sorted cell population will have no 4n peak.



FACS plot depicting 1n, 2n, and little to no 4n peak

Sort into conditioned media before transferring to 10cm plate for outgrowth. Expect relatively large volumes of sort buffer in output tube, so gentle homogenization and supplementation of extra media in outgrowth plates is recommended.

It is not recommended to pellet and resuspend recently sorted cells.

Replace growth media with fresh growth media 24hr after sorting.

9 Verification 2w

After sufficient outgrowth (Typically 50-100 vials of 10 million cells per vial), perform a ploidy check on a population of grown cells.

We recommend checking cells that have been frozen, thawed, and allowed 1 passage before verifying ploidy, as this mimics the pre-transfection conditions for Saturation Genome Editing experiments.