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Tissue culture- Purity sorting HEK293T LLP iCasp9 cells

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Abstract

This protocol covers the sorting of landing pad cells (HEK293T-LLP-iCasp9-blast) for purity prior to starting VAMPseq experiments. This avoids using cells which have undergone landing pad silencing, and increases the proportion of cells that are recombined during transfection experiments. Cells are sorted on blue fluorescence, as cells which do express the landing pad also express blue fluorescent protein (BFP).

Materials

Materials:

- Growing culture of HEK293T-LLP-iCasp9-Blast (Matreyek et al. 2020)
- Falcon® Round-Bottom Tubes Disposable Polystyrene Corning® 5 mL **Corning Catalog #**352054
- DPBS no calcium no magnesium **Gibco - Thermo Fischer Catalog #**14190250
- Flow buffer

1. DPBS 1X
2. 0.5-1.0% Bovine Serum Albumin (Sigma, Catalog # A2153-100G)

- Trypsin-EDTA (0.05%), phenol red **Thermo Fisher Catalog #**25300054
- D10 media

1. DMEM, high glucose **Thermo Fisher Catalog #**11965118
2. Fetal Bovine Serum, Tet system approved, USDA-approved regions **Thermo Fisher Scientific Catalog #**A4736401
3. Penicillin-Streptomycin (10,000 U/mL) **Thermo Fisher Scientific Catalog #**15140122)

- TC-treated Cell Culture Flask 75cm2 **Thomas Scientific Catalog #**1194Z07
- Doxycycline
- 10mg/ml Blasticidin **InvivoGen Catalog #**ant-bl-1
- 1.7 ml microcentrifuge tube **Thomas Scientific Catalog #**1159M35
- Trypan Blue Solution 0.4% **Thermo Fisher Scientific Catalog #**15250061
- Hemocytometer
- 15-mL conical tubes, sterile **VWR International Catalog #**89039-664

Equipment:

- Centrifuge
- Aspirator
- Flow cytometer capable of sorting by fluorescence (e.g. BD FACSAria II)
- Microscope

Before start

- Ensure that the culture of cells has been growing for several days before purity sorting - do not use freshly thawed cells in this protocol.
- Cells should be sorted using an 85-um nozzle for a 4-way sterile purity sort (these are flow cytometer settings).

Preparation of cells

1 Remove media from flask by aspiration.

2 Wash flask with 10 mL DPBS. Remove DPBS by aspiration.

3 Add 3 mL trypsin to the culture flask. Place in a 37 °C incubator with 5% CO_2 for 00:05:00 . 5m

4 Visualize trypsinized cells under a microscope to confirm cells have detached.

5 Add 7 mL D10 media and rinse flask with this media to collect all cells. Transfer this 10 mL of media and cells to a 15-mL conical tube.

6 Centrifuge at 300 x g, 00:05:00 . 5m

7 Aspirate off media and resuspend the pellet in 5 mL of flow buffer.

8 Save 1 mL as a backup in case the sort fails or the sorted cells become contaminated. Centrifuge these cells again, aspirate the media, and resuspend in 1 mL D10 media. Plate in a T-75 flask.

9 Transfer the remaining 4 mL of resuspended cells (in flow buffer) to two 5-mL round bottom polystyrene test tubes (from here called flow tubes).

10 Prepare several flow tubes for recovery of sorted cells - label and fill with 2 mL D10 media.

Sort for purity

11 On the flow cytometer software, open your experiment and create a tube within that experiment for the cells.

12 Remove the cap and load the flow tube containing cells into the sample loader. Press “Load” on the software. Analysis should begin automatically, or press “Acquire data”.

13 Gate for single cells by gating in the following order:

- FSC-A vs SSC-A to gate for live cells
- FSC-A vs FSC-H to gate for single cells in the forward direction
- SSC-A vs SSC-H to gate for single cells in the side direction

14 Create a graph that shows BFP fluorescence on the x-axis and choose “histogram” on the y-axis. Adjust the BFP laser setting if needed so that the main peak (with strong BFP fluorescence) is centered over 10^5.

15 Gate for cells that fluoresce BFP - select a tight area around the positive peak. This will be the sorted population. Note the percentage of the single cell population that expresses BFP. Click on “Record data”.

- If the percentage is >98%, sorting is not necessary.

16 Install the recovery tube in the sorter.

17 In the software’s experiment dashboard, open the “Cytometer settings”, then open a sort layout.

18 Choose the stall which contains the recovery tube, and select the population to sort into the recovery tube (the BFP-expressing cells gated in step 15).

19 Click on the “Sort” button in the sort layout window.

20 As the flow cytometer sorts the cells, pay attention to the volumes in both the original flow tube and the recovery tube. Do NOT let the original flow tube run empty, or the recovery tube overflow.

- To stop the sorter, click on “Pause” in the sort layout window - this does not stop the sorter from pulling from the original flow tube.
- Click on “Stop Acquisition” or “Unload” if it is necessary to change the tube on the sample loader.

21 Sort cells into the prepared recovery tube until the total number of cells needed is reached.

22 Remove tubes from the cytometer and follow instructions for post-sorting cleanup given by the manager of the flow cytometer.

Plating sorted cells

5m

23 Centrifuge the recovery tubes at 300 x g, 00:05:00 . 5m

24 While the centrifuge is running, prepare a new flask for the desired volume of culture by adding N-1mL to the flask (for a desired 10 mL culture, add 9 mL to the flask now). Add doxycycline and blasticidin to the flask.

25 Aspirate the supernatant from the recovery tubes.

26 Resuspend the cells in a total of 1 mL D10 media (for 2 recovery tubes add 500uL to each, etc.).

27 Add the resuspended cells to the prepared flask.

28 Optional: estimate the number of cells by staining the cells with trypan and counting using a hemocytometer.

29 Place the flask in a 37 °C incubator with 5% CO2 and check cells until confluence.

Protocol references

1. Matreyek KA, Stephany JJ, Chiasson MA, Hasle N, Fowler DM. An improved platform for functional assessment of large protein libraries in mammalian cells. Nucleic Acids Res. 2020 Jan 10;48(1):e1. doi: 10.1093/nar/gkz910. PMID: 31612958; PMCID: PMC7145622.