Harvesting and CH_3CN quenching of adhered cells, suspended cells; CH_3CN quenching of tissue by Teresa Fan, University of Kentucky

ADHERED CELLS

Note: This procedure follows [Fan_Cell_Tracer_Ex]. Step 6 from that SOP is detailed here.

PREPARATION

1. Cells: Cell density in the plate should be about 80-95% confluence in a 10 cm cell culture plate
2. Cold CH_3CN: Cool down a bottle of 100% CH_3CN (Optima or HPLC grade) in -20°C freezer overnight before extraction.
3. Ice tray, put following items in the tray
    a. Cold non-sterile PBS and cold CH_3CN
    b. A new 15 ml screw-cap conical tube (Sarstedt, 62.554.002)
    c. A cell lifter (Fisher, 11577692).There is no need to be sterile, so a used but clean one is fine)
    d. The plate(s) with cells

PROCEDURE (All operations should be performed on ice to reduce metabolite degradation. Fill a plastic bag with ice to provide a clear surface. This helps to prevent ice or water from jumping into the plate.

1. Medium removal and Wash (use a vacuum line to suck up cell culture medium and other liquids)
    a. Transfer media into a 15 ml conical tube, centrifuge at 3,500xg for 20 min, 4°C (can be done along with the cell extract below). Remove supernatant and aliquot 100 µl in a 2 ml screw top tube (USA Scientific 1420-8700) for metabolite extraction and freeze remaining media at -80°C for exosomal isolation.
    b. Add 5 ml of cold non-sterile PBS buffer onto the plate, and gently rotate the plate to wash all surface of the plate. Remove PBS by vacuum-suction.
    c. Repeat step (b) twice and remove as much PBS as possible after the 3rd wash to minimize salt contribution to the extract:
        i. Let the plate sit tilted on ice for about 1 min. Keep aspirating liquid in the bottom corner without touching the cells.
2. Quenching and collection
    a. Add 1 ml of cold CH_3CN to cover the whole plate and let stand for ≥ 5 min (can be put at -20C, helps with the cell lysis)
    b. Add 0.55 ml nanopure water + 0.2 ml 0.2 mM Tris-HCl pH 8 as an internal standard for NMR; this is optional) to the plate.

    Note: Prepare a “mastermix” to make sure every sample gets the same concentration of Tris (add water/Tris just before scraping the cells: water will facilitate vigorous scraping with cell lifter and prevent plastic from coming off the plate in CH_3CN)
    c. Scrape cells, and collect cells into a 15 ml polypropylene centrifuge tube (see Fig. 4)

i. Scrape the surface of the plate using the cell lifter. Do not forget to scrape the edge of the plate.
ii. Let the plate sit tilted on ice, and use the lifter to push all cells and CH_3CN/H_2O into the bottom corner.
iii. Collect the cells and the CH_3CN/H2O into the 15 ml tube using a transfer pipette.

d. Repeat step (a) to (c) to collect cells again except that 0.75 ml nanopure water (no Tris) is used.

Note: The final CH_3CN to water ratio is 2:1.5 (v/v)

e. Combine both collections into the 15 ml tube.

Note: This is a break point; as soon as the chloroform is added the procedure has to be completed. Chloroform will eventually dissolve the plastic of the 15 mL tube, but it is fine for short-term use.

SUSPENDED CELLS

1. Centrifuge cells @ 281xg, 4°C for 5 min to pellet the cells
2. Collect the medium supernantant, save 0.1 ml in a 1.5 ml microfuge tube and also save 1.5 ml in a separate 2 ml screw cap tube for safekeeping at -80C
3. Resuspend the cell pellet in 10 ml ice-cold PBS and centrifuge at 281xg, 4°C, 5 min
4. Remove as much PBS wash as possible
5. Resuspend the cell pellet in 0.5 ml ice cold PBS and transfer the suspension to a pretared 1.5 ml microfuge tube (record the tare wt); wash the cell tube with another 0.5 ml ice cold PBS and pool with the 1st 0.5 ml suspension
6. Centrifuge the pooled cell suspension at 1700xg/4°C/5 min to hard pellet the cells
7. Remove as much as possible PBS (this is an important step to minimize salt and to get more accurate wet wt. You can use a gel loading tip at the end of a vacuum line to suck up the buffer or use an ultra-fine pipet tip to remove PBS. The former method is preferred)
8. Immediately after removing PBS, wipe out any moisture on tube exterior, weigh the tube, and flash-freeze in liq N2.

TISSUE QUENCHING

1. Add 2 ml of cold CH_3CN to cover the frozen tissue power (pulverized in liq. N_2 TO fine powder) in 15 ml polypropylene conical centrifuge tube (Sarstedt) and let the powder thaw on ice.
2. Add 0.75 ml nanopure water + Tris-HCl (pre-mixture of 0.55 ml nanopure water + 0.2 ml 0.2 mM Tris-HCl (pH 8)) (as internal standard; this is optional) to the tube.
3. Add 0.75 ml nanopure water (without Tris) to the tube and vortex rigorously to maximize extraction.

Note : total amount of water should be 1.50 ml and the ratio of CH_3CN to water should be 2 : 1.5.