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WP3

Task 3.2

Protocol for CD133 cell surface staining (Antibody anti-Human CD133 130-098-826 MACS)

The recommended antibody dilution for labeling of cells and subsequent analysis by flow cytometry is 1:10 for up to 10^7 cells/100 μ L of buffer.

N.B. - Volumes given below are for up to 10^7 nucleated cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^7 nucleated cells, use twice the volume of all indicated reagent volumes).

1. Determine cell number (10^7).
2. Centrifuge cell suspension at 1000 RPM for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to 10^7 nucleated cells per 100 μ L of buffer (Buffer: Prepare a solution containing phosphate buffer saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA). Keep buffer cold (2–8 °C).
4. Add 10 μ L of the antibody.
5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).

Note: Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice requires increased incubation times.

5. Wash cells by adding 1–2 mL of buffer and centrifuge at 1000 RPM for 10 minutes. Aspirate supernatant completely.
6. Resuspend cell pellet in PBS for analysis by flow cytometry.

Glomax protocols

-The CellTiter-Fluor™ Cell Viability Assay (G6081, Promega) is a nonlytic, single-reagent-addition fluorescence assay that measures the relative number of live cells in a culture population after experimental manipulation. The CellTiter-Fluor™ Cell Viability Assay measures a conserved and constitutive protease activity within live cells and therefore serves as a marker of cell viability. The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant, peptide substrate (glycyl-phenylalanyl-aminofluorocoumarin; GF-AFC). The substrate enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium.

Reagent Preparation and Storage

1. Completely thaw the CellTiter-Fluor™ Cell Viability Assay components in a 37°C water bath. Vortex the GF-AFC substrate to ensure homogeneity, then briefly centrifuge for complete substrate volume recovery.
2. Transfer the GF-AFC Substrate (10µl for Cat.# G6081) into the Assay Buffer container (10ml for Cat.# G6081) to form a 2X Reagent. Mix by vortexing the contents until the substrate is thoroughly dissolved. Note: The solution may initially appear “milky” when the GF-AFC substrate is delivered to the buffer. This is normal. The substrate will dissolve with vortexing.
3. Add the reagent at 1/1 the volume of the cell culture (i.e., 100µl in 100µl di cell culture).

Storage: The CellTiter-Fluor™ Viability Reagent should be used within 24 hours if stored at room temperature. Unused reconstituted CellTiter-Fluor™ Viability Reagent can be stored at 4°C for up to 7 days with no appreciable loss of activity.

The CellTiter-Fluor™ Reagent may be scaled to accommodate the volumes required for downstream multiplexes. To do this, use 1/5 the volume of buffer when you prepare the reagent (i.e., 10µl of the GF-AFC Substrate in 2ml of Assay Buffer), add the reagent at 1/5 the volume of the cell culture (i.e., 20µl in 100µl di cell culture).

Viability Assay Protocol

1. Expose cells to EMFs in appropriate cuvette (100000cells) and/or in combination with RX (2-5-8Gy of x-Rays)

2. Settle cells in each well at desired density (20000 cells for D283; 10000 cells for Daoy) in 96- well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom) containing culture medium (the final volume is 100µl)
3. Culture cells for the desired test exposure period (4-16-24h)
4. Add CellTiter-Fluor™ Reagent in an equal volume (100µl per well) to all wells, mix briefly by orbital shaking, then incubate for at least 30 minutes at 37°C.

Note: Longer incubations may improve assay sensitivity and dynamic range. However, do not incubate more than 3 hours, and be sure to shield plates from ambient light (we used 1.5h).

5. Measure resulting fluorescence using a fluorometer (380–400nmEx/505nmEm).

-The Caspase-Glo® 3/7 Assay (G8093, Promega) is a homogeneous, luminescent assay that measures caspase-3 and -7 activities. These members of the cysteine aspartic acid-specific protease (caspase) family play key effector roles in apoptosis in mammalian cells. The assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. Adding a single Caspase-Glo® 3/7 Reagent in an “add-mix-measure” format results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present.

Reagent Preparation and Storage

1. Equilibrate the Caspase-Glo® 3/7 Buffer and lyophilized Caspase-Glo® 3/7 Substrate to room temperature before use.
2. Transfer the contents of the Caspase-Glo® 3/7 Buffer bottle into the amber bottle containing Caspase-Glo® 3/7 Substrate. Mix by swirling or inverting the contents until the substrate is thoroughly dissolved to form the Caspase-Glo® 3/7 Reagent. Buffer volume is 10ml for G8093.

Storage: The reconstituted Caspase-Glo® 3/7 Reagent may be stored at 4°C for up to 3 days with no loss of activity compared to that of freshly prepared reagent. Reconstituted reagent stored at 4°C for 1 week will give a signal approximately 90% of that obtained with freshly prepared reagent, while reconstituted reagent stored at 4°C for 4 weeks will give a signal approximately 75% of that obtained with freshly prepared reagent. Reconstituted reagent that has been refrozen and stored at –20°C for 1 week will give a signal approximately 75% of that of freshly prepared reagent, and refrozen reagent stored at –20°C for 4 weeks will give a signal approximately 60% of that of freshly prepared reagent.

Caspase-3 and -7 Activities in Cell-Based Assays

1. Expose cells to EMFs in appropriate cuvette (100000cells) and/or in combination with RX (2-5-8Gy of x-Rays)

2. 96- well white-walled multiwell luminometer plates compatible with luminometer containing cells in culture medium (the final volume is 100µl) in each well at desired density (20000 cells for D283; 10000 cells for Daoy).
3. Culture cells for the desired test exposure period (4-16-24h)
4. Add an equal volume of Caspase-Glo® 3/7 Reagent to sample volume (1:1 ratio; 100µl per well) to all wells, mix briefly by orbital shaking, then incubate for at least 30 minutes to 3hr at room temperature, then measure luminescence using a luminometer.

- Multiplex Assay Protocol (Viability assay with luminescent Caspase Assay)

1. Expose cells to EMFs in appropriate cuvette (100000cells) and/or in combination with RX (2-5-8Gy of x-Rays).
2. Settle cells, at the desired density, in 96-well assay plates containing culture medium (final volume is 100µl in each well).
3. Culture cells for the desired test exposure period.
4. Add 20µl of CellTiter-Fluor™ Reagent (prepared as 10µl substrate in 2ml Assay Buffer) to all wells, and mix briefly by orbital shaking. Incubate for at least 30 minutes at 37°C. Note: Longer incubations may improve assay sensitivity and dynamic range. However, do not incubate longer than 3 hours, and be sure to shield plates from ambient light.
5. Measure resulting fluorescence (viability) using a fluorometer (380–400nmEx/505nmEm).
6. Add an equal volume of Caspase-Glo® 3/7 Reagent, prepared as described, to wells (100–120µl per well), incubate for at least 30 minutes to 3hr at room temperature, then measure luminescence using a luminometer.