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UABMC - Stemness Determination Protocol

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1 Works for me This protocol is published without a DOI.

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ABSTRACT

This procedure describes the assays for determining stem-ness in the PDMC models.

The phenotypic and marker-based assays that can and will be used for determining/classifying the stem-cell like characteristics of a particular model, namely neurosphere, geltrex (or equivalent) adherent cells, or microtumors.

Several of these assays are not gold-standard but are semi-validated surrogates for stem-like cells

PROTOCOL CITATION

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GUIDELINES

U01 Subgroup:
Derivative Models group (Neurospheres and Microtumors)

Approvers:
Anita Hjelmeland

Responsibilities:
Research Technician will perform these studies

MATERIALS

NAME	CATALOG #	VENDOR
Penicillin-Streptomycin (5,000 U/mL)	15070063	Thermo Fisher
Bovine Albumin Fraction V (7.5% solution)	15260037	Gibco, ThermoFisher

MATERIALS TEXT

CD133/1 (AC133)-APC, human
- for 100 tests Miltenyi 130-113-106
- for 30 tests Miltenyi 130-113-668
This antibody is expensive and does not last long, so make sure to order the appropriate amount.

CD15-FITC, human
for 100 tests Miltenyi 130-113-484
for 30 tests Miltenyi 130-114-010

5% BSA solution:Add 6.6 mL of 7.5% BSA (ThermoFisher 15260037), 3.4 mL naught Media and 100 µL pen/strep (ThermoFisher 15070063).

SAFETY WARNINGS

1. All human-derived tumor specimens are handled under BSL2 conditions. Work is conducted in the BSC using personal protective equipment and avoiding the use of sharps where possible.
2. All materials potentially exposed to human material are treated with a 10% bleach solution for a minimum of 10 minutes, double bagged for autoclaving, or incinerated.

LIMITING DILUTION ASSAY

1 Ensure cultures are healthy with minimal percentages of dead cells

2 Dissociate cells using Accutase as usual

3 While cells are dissociating, add stem cell media to inner wells (6x10) at 200uL per well and to well H2. Add water to the outer wells at 300uL per well except for well H2.

4 Add PI stain to the dissociated cells

5 Take to flow cytometry and sort PI- cells into 96 wells

6 Plate cells in limiting dilution down to 1 cell per well.

7 A good starting template for stem cell enriched cultures is:

B2-B11	1 cell per well
C2-C11	5 cells per well
D2-D11	10 cells per well
E2-E11	25 cells per well
F2-F11	50 cells per well
G2-G11	100 cells per well
H2	1000 cells (+ control to adjust visual plane)

A good starting template for bulk cultures is:

B2-B11	1 cell per well
C2-C11	20 cells per well
D2-D11	50 cells per well
E2-E11	100 cells per well
F2-F11	500 cells per well

G2-G11	1000 cells per well
H2	5000 cells (+ control to adjust visual plane)

8 For repetitions, adjust as needed based on prior results by increasing the number of wells in the ranges where the sphere formation is less than 100% but greater. Once you have identified working conditions, increase the # of wells to be at least two rows (20 wells) of each to gain statistical power. than 0%. Keep numbers so that one row is expected to have 100% spheres.

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If the above method has not worked previously, save the conditioned media from the cells prior to dissociation.

1. Filter through a 0.45 micron filter and then plate this media into the 96 wells with 100uL conditioned media and 100uL fresh media per well.
2. If flow cytometry is a problem due to either inaccurate cell numbers (check immediately post flow), viability post flow (check 24hrs post flow) or contamination, you can plate the assay by hand.
3. In this case, if you are comparing two cell types (rather than completing a drug treatment) you must count the cells in at least triplicate prior to initiating the assay to ensure correct counts.
4. Dilute cells to 5000 cells/200uL= 250000 cells/10 mL and plate well H2. Then dilute from this stock in a stepwise fashion as needed: ie 5000 cells/200uL to 1000 cells/200uL. Then 1000 cells/200uL to 500 cells/200uL etc.

10 Check plates at 7, 14, and 21 days. Note the number of wells with spheres then use this information along with the total number of wells and cells plated to complete Statistical Analysis at http://bioinf.wehi.edu.au/software/elda/

11 At an optimal time point for one cell number condition (need condition where spheres are individual and no merging has occurred, for example 2nd lowest cell number where spheres formed at 14 days), can also collect data for the number of spheres per well as well as the size of the spheres formed. However, these data are not as important for publication as the limiting dilution analysis.

MARKER ASSESSMENT

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For analysis only. If sorting for propagation, MACS rather than FACS is recommended.

If Miltenyi tumor dissociation kits are used, cells can be sorted immediately. If Papain is used for tumor dissociation, ensure that cells have recovered at least **06:00:00** but preferably **Overnight** . If cells in culture are dissociated with Accutase, ensure that cells have recovered at least 6 hrs but preferably overnight. Regardless of the tissue dissociation protocol, ensure cells are healthy prior to beginning.

13 Ensure cells are single cells for flow. If flow is not performed shortly after dissociation, triturate cells by passing through a P200 tip at least 20 times and then pass through a 40 um filter.

14 Wash cells in naught media, count cells and resuspend in **400 µl** naught media and **400 µl** FcR Blocking Reagent for every 10 million cells.

15 Distribute cells to appropriate tubes for no staining, viability dye alone, isotype control along, and antibody and viability labeling. For the first three tubes, only a very small portion of cells are required (at least 100,000 cells).

16 When using the antibodies, ensure the hood light is off and tubes are covered in aluminum foil/kept in the dark.

17 Add the antibodies or appropriate controls. As Milteny isotype antibodies are **20 ug/mL** , use **1 µl** of these isotype controls. Add **50 µl** of CD133/2 (293C3)-APC antibody for up to 10 million cells. Mix by shaking the tube and then incubate for **00:30:00** at **Room temperature** , mixing by shaking the tube every 5 min.

18 After the **00:30:00** , wash the cells at **1000 rpm** with **3 mL** of naught media for **00:05:00**

19 Resuspend the cells in 5% BSA with pen/strep. Use **500 µl** for 10 million cells or **200 µl** for controls.