Macrophage Cell Retention/Release

(April 2020)

Introduction

This protocol describes macrophage retention and release of Nano formulations.

Definitions

DPBS: Dulbecco's Phosphate Buffered Saline

Reagents and Materials

- Plated cells in Falcon 12-well flat bottom tissue culture plate with low evaporation lid; Plate Label _____
  Manufacture: Fisher Scientific

- Drug Formulations:
  - Formulation name _____,
    Manufacture Date _____, Concentration (mg/mL) _____
  - Formulation name _____,
    Manufacture Date _____, Concentration (mg/mL) _____
  - Formulation name _____,
    Manufacture Date _____, Concentration (mg/mL) _____
  - Formulation name _____,
    Manufacture Date _____, Concentration (mg/mL) _____

- Media B
  Note: made in house via Media B protocol
  Manufacture date _____

- Gibco DPBS (Dulbecco's Phosphate Buffered Saline) (1X) (- Calcium Chloride, - Magnesium Chloride)
  Day 5 Lot # _____
  Day 10 Lot # _____
  Day 20 Lot # _____
  Day 30 Lot # _____
  Manufacturer: Gibco

- Fisher Disposable Cell Lifter
  Manufacturer: Fisher Scientific

- Trypan Blue (0.4%)
  Manufacturer: Fisher Scientific

- HPLC grade Methanol
  Day 5 Lot # _____

Day 10 Lot # _____
Day 20 Lot # _____
Day 30 Lot # _____
Manufacturer: Fisher Scientific

- Cole-Parmer Ultrasonic Processor with microtip sonication probe;
  SN _____
  Manufacturer: Cole-Palmer

- Round bottom culture plates, 96 well, low evaporation, sterile
  Manufacturer: Fisher Scientific

- Rubber mat, 96-well plate cover
  Manufacturer: Fisher Scientific

Instrumentation
Cell Incubator (37 °C, 5%, CO2 standard requirements)
Manufacturer: Fisher Scientific

Invitrogen Countess automated cell counter
Manufacturer: Invitrogen

Countess Cell Counting Chamber Slide
Lot#: _____
Manufacturer: Invitrogen

Bench top Centrifuge
Manufacturer: Fisher Scientific

Methods –

1. Warm Media B to 37 ℃ in water bath (20 mL/plate(treatment))
2. Dilute formulations to appropriate concentration with warmed Media B; Treatment Concentration _____
3. Remove old Media from cells
4. Quickly, yet carefully, add 1.5 mL/well treated Media to each well
5. Place in incubator (37 ℃, 5% CO_2) for 8 hour treatment
6. After 8 hours, remove treated Media from cells
7. Carefully wash each well 2X with 1 mL PBS
8. Carefully add 1.5 mL/well warmed, fresh Media B to each well; cover and place in incubator until ready to collect media/cells
9. Maintain cells by doing a full media change with warmed, fresh Media B as needed (remove and save old Media in labeled 1.7 mL centrifuge tubes for drug release studies and replace with 1.5 mL fresh Media B)

10. On each collection day (D5, D10, D20, and D30 post-treatment), remove and collect media from cells into labeled 1.7 mL centrifuge tubes
11. Carefully wash each well 2X with 1 mL PBS
12. Add 1 mL PBS
13. Scrape cells with disposable cell lifter
14. Collect suspended cells into labeled centrifuge tubes for retention studies
15. Count cells in one representative well for each treatment by mixing 10 uL cell suspension with 10 uL Trypan Blue in centrifuge tube; pipet up and down to thoroughly mix
16. Add 10 uL mixture to cell counting chamber slide
17. Place slide in cell counter
18. Adjust focus
19. Count cells
20. Spin cell suspension at 3,000 rpm for 8 minutes at 4 °C
21. After centrifugation, carefully remove and discard supernatant (being careful not to disrupt cell pellet)
22. Add 200 uL fresh HPLC grade Methanol to each tube; At this point samples (both cells samples and media samples) may be stored at -80 °C for later analysis or they may be processed for immediate analysis

Cell Counts (Live cells; 10^5 cells/mL)				
	Treatment Time Points (days)			
Treatment: (formulation name)	5	10	20	30

For cell sample processing (Retention studies) –

1. If samples were stored; Remove samples from -80 °C, let warm to Room Temperature
2. Sonicate samples for 2 seconds each using the sonication probe; clean probe with 70% Ethanol then 100% Methanol between each sample, wipe dry with Kim Wipe
3. Briefly vortex each sample to ensure well mixed
4. Spin samples at 14,000 rpm for 10 minutes at 4 °C
5. Prepare standard curve for UPLC (see UPLC stds protocol)
6. Load 75 uL of each sample into 96-well plate
7. Carefully cover plate with rubber mat; avoid any cross contamination of samples
8. Place plate in UPLC-UV to be run

For media sample processing (Release studies) –

1. Remove samples from -80 °C, let warm to Room Temperature
2. Briefly vortex samples to ensure well mixed
3. Combine 150 uL of media with 1 mL of HPLC grade Methanol in 1.7 mL centrifuge tube
4. Place samples in -20 °C for 30 minutes
5. Spin samples at 14,000 rpm for 10 minutes at 4 °C
6. Transfer supernatants to new 1.7 mL centrifuge tubes (pellet may be discarded)
7. Place tubes with supernatants in speed-vac until dry (to speed drying, 100-200 uL HPLC grade Ethanol can be added once supernatant volume reaches approximately 100-300 uL)
8. Once media pellet is dry, resuspend in 150 uL of HPLC grade Methanol
9. Briefly vortex samples to ensure well mixed
10. Sonicate samples in sonication water bath for 6 minutes
11. Briefly vortex sample to ensure will mixed
12. Spin samples at 14,000 rpm for 10 minutes at 4 °C
13. Prepare standard curve for UPLC (see UPLC stds protocol)
14. Load 75 uL of each sample into 96-well plate
15. Carefully cover plate with rubber mat; avoid any cross contamination of samples
16. Place plate in UPLC-UV to be run