GENProtocols

Protocol

IsoCode Single-Cell Innate Immune: Human Monocyte Protocol Using Membrane Stain

CANCER RESEARCH DRUG DISCOVERY OMICS COVID

IsoPlexis Applications Team, IsoPlexis

Aug 06, 2021

Abstract

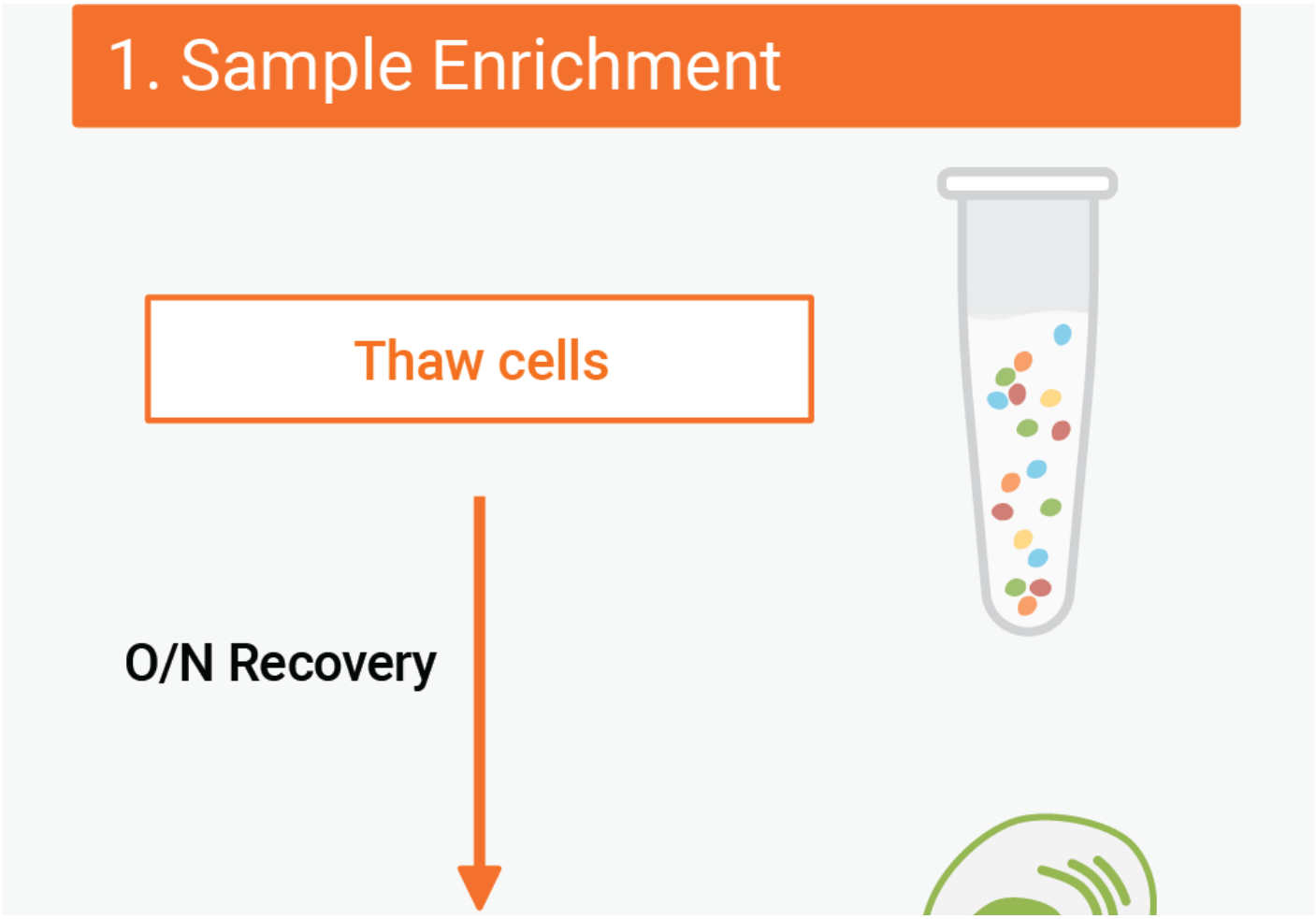
A. Overview

Overview of Protocol

Day 1: Cryopreserved cells are thawed and cultured overnight in complete RPMI media.

Day 2: Enrichment and Stimulation of Monocytes for 24 hours.

Day 3: Staining and Loading of Monocytes onto IsoCode Chip.



Enrich for Monocytes

Monocyte

2. Cell Stimulation

LPS Stim

LPS Stim on plate

24 hrs

Stimulated Monocyte

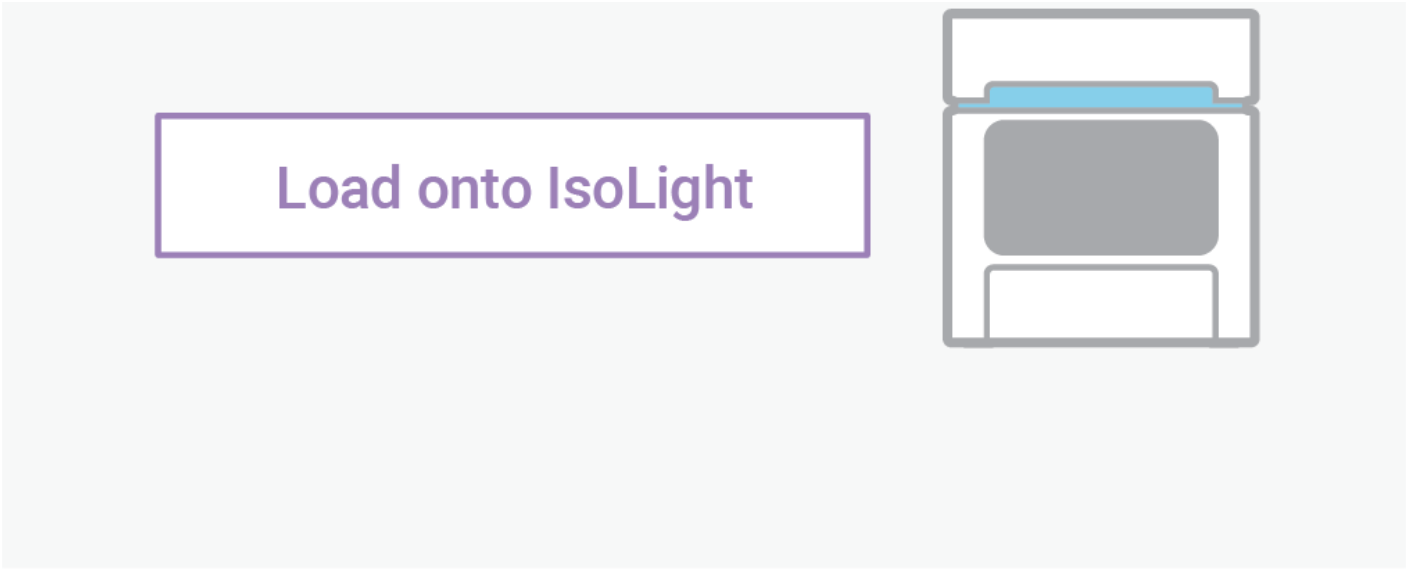
3. Cell Staining

Add Membrane Stain to Monocytes

10 min

Monocyte

4. Load Cells



Introduction

NOTE: This protocol outlines the standard method for thawing and culturing of human monocytes only and may not be valid for other species or cell types.

NOTE: Using stains and protocols other than the included kit surface stains and protocols might result in failed runs. Stains and staining procedures not approved by IsoPlexis will require validation prior to use. Please consider IsoPlexis' IsoPACE™ program to assist in custom marker and protocol validation.

Safety Warnings: Read MSDS documents of all materials prior to use. Laboratory workers should wear standard PPE, including disposable gloves, protective eyewear, and laboratory coats.

Reagents and Equipment

Required Consumables

Table 1: Required Consumables Provided by IsoPlexis

Item	Catalog Number	Quantity	Comment
IsoCode Kit	Please see website (https://isoplexis.com/) for available kits or talk to IsoPlexis' Customer Service team for details	One chip per sample/cell type/condition	Subcomponents stored at 4°C and -20°C

IsoCode Kit Components

- **IsoCode Reagent Box (4°C)**
  - 15 mL Tube A
  - 15 mL Tube B
  - 1.5 mL Tubes A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
  - 50 mL Tubes containing Reagents 1, 2, 3, 4, 5, 6, 7, 8
  - 1 Bag of Disposable Reagent Sippers
- **IsoCode Chip Set (-20°C)**
  - Boxes of IsoCode Chips (2 per box) to make up 4, 6, or 8 chips

- Membrane Stain [ordered separately]
- Membrane Stain Diluent (DMSO) [ordered separately]

Table 2: Consumables Required to Perform Protocol but Not Supplied by IsoPlexis

Consumable	Type	Source	Catalog Number
T25 Flask	N/A	Corning	353108
T75 Flask	N/A	Corning	430641U
48 well flat bottom plate	N/A	Corning	3548
24 well flat bottom plate	N/A	Corning	3524
6 well flat bottom plate	N/A	Corning	3516
MACS LS Column	N/A	Miltenyi	130-042-401
Falcon Centrifuge Tubes	Polypropylene, 15 mL	VWR	CA62406-200
Pipette tips (filtered)	10 µL Graduated Filter Tips 100 µL Graduated Filter Tips 1000 µL XL Graduated Filter Tips	USA Scientific	1181-3710 1183-1740 1182-1730
Serological Pipette	2mL Pipette 5 mL Pipette 10 mL Pipette	USA Scientific	1072-0510 1075-0110 1071-0810
Lo-bind Microcentrifuge tubes, sterile	1.5 mL	Eppendorf	4043-1081
Cell scraper (depending on plate size)	N/A	Corning	3010
Mini cell scraper (depending on plate size)	N/A	Biotium	22003

Table 3: Required* Reagents Not Supplied by IsoPlexis

Consumable	Stock Concentration	Source	Catalog Number
RPMI	1x	Fisher	MT10040CV
Penicillin-Streptomycin-Neomycin Solution Stabilized	100x	Sigma	P4083-100mL
Glutamax	100x	Thermo	35050061
FBS	1x	Sigma	F2442-6X500mL
Phosphate buffered saline (1XPBS) without Calcium or Magnesium	1x	Invitrogen	20012-027
RoboSep buffer	1x	StemCell Tech	20104
Ficoll Paque Plus	N/A	GE Healthcare	17-1440-03
Miltenyi Pan Monocyte Kit	N/A	Miltenyi	130-096-537
Trypan Blue	0.04%	Gibco	15250-061
Accutase	1x	Innovative Cell Technologies, Inc	AT 104-500
LPS (lyophilized powder)	N/A	Sigma	L2654-1MG
Reagent alcohol 70%	N/A	VWR	BDH1164-4LP

Table 4: Cell Staining Reagents

Test Material	Catalog Number	Color
Membrane stain	STAIN-1001-1	Violet

Table 5: Required Equipment

Equipment	Source	Catalog Number/Requirements
IsoLight Instrument	IsoPlexis	ISOLIGHT-1000-1
Culture Hemocytometer	(Fisher) Hauser Levy	02-671-55A
Hemocytometer Cover Glass	(Fisher) Hauser Levy	02-671-53
MidiMACS separator	Miltenyi	130-042-302
MACS MultiStand	Miltenyi	130-042-303

Table 6: General Equipment

Equipment	Requirements
Pipette	P10, P100, P200, P1000
Pipettor	Ability to pipette between 1 and 10 mL
Incubator	37°C, 5% CO_2
Tabletop Centrifuge	Temperature controlled*; swinging bucket rotor; ability to centrifuge 15 mL and 50 mL conical tubes
Microcentrifuge	Temperature controlled*; fixed rotor; ability to centrifuge 1.5 mL microcentrifuge tubes
Mini centrifuge	Ability to spin micro sample sizes
Water Bath	Ability to heat to 37°C
Microscope	Inverted light microscope with 10x and 20x objectives

* Temperature controlled centrifuges are required so that centrifuging steps can be conducted at room temperature without risk of overheating. Temperature on centrifuges should be set to 21°C.

Procedure

B. Before Getting Started

1. Important Precautions

Read MSDS documents of all materials prior to use.

Working with Biohazardous Reagents: Please refer to your institute's guidelines and obtain proper training to handle potentially biohazardous samples. It is also strongly recommended that any lab personnel handling human samples be vaccinated against HBV if the individual does not have sufficient HBV antibody titer.

Additional precautions need to be taken when working with samples that potentially contain an EID agent:

1. Laboratory workers should wear standard PPE, including disposable gloves, protective eyewear, and laboratory coats.
2. Any procedure or process that cannot be conducted in the designated EID BSC should be performed while wearing gloves, gown, goggles and a fit tested N-95 mask.
3. Work surfaces should be decontaminated on completion of work with appropriate disinfectants. This includes any surface that potentially came in contact with the specimen (centrifuge, microscope, etc.).
4. All liquid waste produced in the processes must be treated to a final concentration of 10% bleach prior to disposal.

2. Reagents to Be Prepared Before Starting

Table 7: LPS Recipe

CRITICAL: LPS has been validated for use by IsoPlexis. Using alternative stimulant may result in failed runs. Please contact FAS for additional information.

Ingredient	Stock Concentration	Final Concentration	Amount for 1 mL	Vendor/Catalog
LPS	N/A	1mg/mL	1mg	Sigma L2654-1MG
PBS	1x	1x	1mL	Invitrogen 20012-027

- CRITICAL: Prepare 10µL LPS aliquots and freeze at -20°C for no longer than 2 months. Aliquots are single- use only and are to be thawed immediately prior to their usage. If there is any remaining volume in an aliquot, do not refreeze but discard.

Table 8: Complete RPMI Recipe

Critical: Complete RPMI media has been validated for use by IsoPlexis. Using alternative media may result in failed runs. Please contact FAS for additional information.

Ingredient	Stock Concentration	Final Concentration	Amount for 500 mL	Vendor/Catalog
Penicillin-Streptomycin-Neomycin	100x	1x	5 mL	Sigma/ P4083-100mL
Glutamax	100x	1x	5 mL	Thermo/ 35050061
FBS	1x	10%	50 mL	Sigma/ F2442-6X500 mL
RPMI	1x	1x	440 mL	Fisher/ MT10040CV

Note | Sterile-filter through 0.22 µm filter before use. Store complete RPMI Media at 4°C and warm up to 37°C in water bath prior to use.

C. Protocol

Chapter 1: Getting Started

Kit Contents:

- **IsoCode Reagent Box (4°C)**
  - 15 mL Tube A
  - 15 mL Tube B
  - 1.5 mL Tube A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
  - 50 mL Tubes Containing Reagents 1, 2, 3, 4, 5, 6, 7, 8
  - 1 Bag of Disposable Reagent Sippers
- **IsoCode Chip Set (-20°C)**
  - Boxes of IsoCode Chips (2 Per Box) to Make Up 4, 6, or 8 Chips
  - Membrane Stain in Micro-Tube (Blue Cap)

- Membrane Stain Diluent (DMSO) (Brown Cap)

Chapter 2: Recovery of Cryopreserved Cells

Materials Required:

- Complete RPMI (37°C)
- Cryopreserved PBMC
- 15 mL Centrifuge Tube
- T25 Flask or T75 Flask

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 21°C.

Methods:

1. Pipette 5 mL of pre-warmed complete RPMI into a 15 mL centrifuge tube, labeled Thawed PBMC.

2. Using proper PPE, remove cells from liquid nitrogen storage and thaw cells. **TIP: Be careful of contamination.**

3. Quickly move vials into a water bath (37°C) to thaw. While thawing, swirl the vial in the water until a single ice crystal remains in the vial. Be sure to prevent (to the best of your ability) any of the water from the water bath from getting underneath the cap and into the sample.

4. When the sample is nearly thawed, remove the vial and immediately spray vial with 70% alcohol before bringing into the hood. It is important to allow the alcohol to evaporate before opening the vial.

5. Slowly pipette thawed cells into 5 mL of complete RPMI in 15 mL centrifuge tube, labeled Thawed PBMC. **TIP: Insert tip into complete RPMI when pipetting, be careful to not create bubbles.**

6. Take 1 mL of the cell/complete RPMI mixture.

7. Pipette into original thawed cell vial, rinse inside the vial with the complete RPMI to recover additional thawed cells. **TIP: Insert tip into complete RPMI, be careful to not create bubbles.**

8. Draw up cell/complete RPMI mixture and pipette back into the 15 mL centrifuge tube. **TIP: Insert tip into complete RPMI and pipette gently up and down. Be careful to not create bubbles.**

9. Centrifuge cells for 10 minutes at 300 rcf.

10. After cells are centrifuged, check for cell pellet.

11. Aspirate supernatant. **TIP: Be careful not to aspirate cell pellet.**

- Use pipette to remove last bit of supernatant.

12. Resuspend cell pellet in 1 mL of fresh complete RPMI.

- Mix well to resuspend. **TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.**

13. Slowly add additional complete RPMI to a final concentration of 1 x 106 cells/mL.

14. Transfer cell suspension to flask. For 3-5 mL of cell volume, transfer to a T25 flask, and for 8-10 mL of cell volume, transfer to a T75 flask. **TIP: Slowly pipette down the side of the flask as to not create bubbles.**

15. Spread out cell suspension by rocking the plate or flask carefully to fully cover the bottom. **TIP: Be careful to not make bubbles.**

16. Move to incubator for overnight recovery at 37°C, 5% CO2.

Chapter 3: Pre-Sample Enrichment

Materials Required:

- Complete RPMI (37°C)
- Sterile 1X PBS (Room temperature)
- 1X Accutase (Room temperature)
- 50 mL Centrifuge Tube
- Overnight Recovered Cells from Chapter 2 or Fresh PBMC if Working with Fresh Samples
- Lo-Bind Microcentrifuge Tube for Cell Count

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 21°C.

Methods:

1. Remove nonadherent PBMC by gently pipetting out cells in suspension. Save suspension cells for further monocyte enrichment in 50 mL centrifuge tube.

2. Gently rinse flask with 3 mL PBS (T25) or 5 mL of PBS (T75). Remove PBS from flask by gently pipetting up. Combine PBS wash with cell suspension from Pre-Sample Enrichment Step 1.

3. Thaw 1X Accutase at room temperature. Pipet up and down 5 times to ensure it is well mixed.

4. Detach adherent cells from the flask by adding 2 mL 1X Accutase (T25) or 5 mL 1X Accutase (T75)

5. Rock the plate/flask back and forth to ensure the 1X Accutase covers the entire bottom of the well.

6. Incubate the cells with 1X Accutase for 10 minutes at room temperature to allow the cells to detach. During incubation, be sure to rock the plate back and forth about once per minute. Observe the cells under the microscope until they appear detached.

7. If needed, use a cell scraper to remove additional monocytes from the bottom of the flask.

8. Rinse the flask with 5 mL of complete RPMI to remove the monocytes and combine this fraction with the suspension fraction from step 1.

9. Add 5 mL of complete RPMI to the flask and observe under a light microscope to ensure no cells remain. If cells remain, repeat steps 7 through 8.

10. Mix well 5 times with 10 mL serological pipette. **TIP: Be careful not to create bubbles**. Take a 10 µL aliquot of your cells and transfer to a Lo-Bind Microcentrifuge Tube for cell counting. **CRITICAL: See Appendix D1 for cell counting instructions.**

11. Centrifuge combined cell fractions for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells. **CRITICAL: See Appendix D1 for cell counting instructions.**

CRITICAL: If cells are less than 80% viable, proceed to Appendix D2 Dead Cell Depletion Protocol using Ficoll.

Chapter 4: Pan Monocyte Sample Enrichment

Materials Required:

- Complete RPMI (37°C)
- RoboSep Buffer (4°C)
- Miltenyi Pan Monocyte Kit, Human, (4°C)
- MACS LS Column

- Prepared Cells from Chapter 3
- Enrichment Kit:
    - MACS Metal Plate/Magnet Kit
    - 2 x 15 mL Centrifuge Tubes (Waste, Pan Monocyte fraction)
    - Lo-Bind Microcentrifuge Tube for Post-Enrichment Monocytes

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 21°C.

Methods:

1. Remove the centrifuged cells and check for cell pellet.

2. Aspirate supernatant. **TIP: Be careful not to aspirate the cells.**

- Use pipette to aspirate remaining supernatant.

CRITICAL: Use the following volumes according to the number of cells

Cell quantity	RoboSep (4°C)	FcR Blocking Reagent (4°C)	Biotin-Antibody Cocktail (4°C)
Up to 7,500,000	40 µL	10 µL	10 µL
7,500,000-15,000,000	80 µL	20 µL	20 µL
15,000,000-25,000,000	120 µL	30 µL	30 µL

3. Resuspend cell pellet in cold RoboSep and mix well by pipetting up and down 15 times.

4. Add Miltenyi FcR Blocking Reagent and mix well by gently pipetting up and down 5 times.

5. Add Miltenyi Biotin-Antibody Cocktail and mix well by gently pipetting up and down 15 times.

TIP: Be careful not to create bubbles.

6. Incubate in refrigerator (4°C) for 5 minutes.

TIP: Keep RoboSep in refrigerator during enrichment process.

CRITICAL: Use the following volumes according to the number of cells

Cell quantity	RoboSep (4°C)	Anti-Biotin Microbeads
Up to 7,500,000	30 µL	20 µL
7,500,000-15,000,000	60 µL	40 µL
15,000,000-25,000,000	90 µL	60 µL

7. Add RoboSep to 50 mL centrifuge tube.

8. Add Miltenyi Anti-Biotin MicroBeads and mix well by gently pipetting up and down 15 times.

TIP: Make sure to eliminate clumps so that beads are evenly distributed among cells. Be careful not to create

bubbles.

9. Incubate in refrigerator (4°C) for 10 minutes.

TIP: Keep RoboSep in refrigerator during enrichment process.

10. Set up MACS sorting by setting metal plate in tissue culture hood and placing magnet on metal plate. Place LS column securely in magnet with wings facing out and align the 15 mL centrifuge tube labeled "Waste" under the LS column. **CRITICAL: LS Column should not touch the tubes.**

CRITICAL: Cell suspension must be 500 µL before being placed in LS column. If necessary, add RoboSep to cell suspension

CRITICAL: Be careful not to let column dry out. Do not add liquid when there is already liquid in the LS Column.

11. Start with the LS column over the "Waste" tube, add 3 mL of cold RoboSep to LS Column. **CRITICAL: Be careful not to create bubbles or touch sides of LS column. Let all the RoboSep flow through before moving on to the next step. As a reminder, be careful to not let the column dry out.**

12. Unscrew and keep cap for "Pan Monocyte Fraction" tube. NOTE: This is in preparation for next step to ensure the column does not dry out during the transition.

13. When the last drop falls through to the" Waste" tube, move the rack over so the LS column is over the "Pan Monocyte Fraction" tube. **CRITICAL: Be careful not to let column dry. If there is one drop remaining that will not fall, move on to the next step.**

14. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.

15. Mix cell suspension by gently pipetting up and down 15 times. NOTE: This ensures that the cells are evenly dispersed after sitting.

16. Draw up all 500 µL of cell suspension and pipette carefully into the center of the LS column without touching the sides of the column.

17. Wash column 3 times with 3 mL of cold RoboSep.

- First wash: Rinse inside walls of cell suspension tube with 3 mL of cold RoboSep before transferring the mixture to LS Column. NOTE: This is to retrieve any cells that have been left behind.
    - Pipette all the mixture into LS Column after last drop passes through or does not fall from step 16. **CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.**
- Second wash: Add 3 mL of RoboSep into LS Column after last drop passes through or does not fall. **CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.**
- Third wash: Add 3 mL of RoboSep into LS Column after last drop passes through or does not fall. **CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.**

18. Centrifuge "Pan Monocyte Fraction" tube for 10 minutes at 300 rcf.

19. After cells are centrifuged, check for cell pellets.

20. Aspirate RoboSep buffer from "Pan Monocyte Fraction" tubes. **TIP: Be careful to not aspirate cell pellet.**

21. Use pipette to aspirate the remaining supernatant from each tube. **TIP: Be careful to not aspirate cell pellet.**

22. Add 1 mL complete RPMI to "Pan Monocyte Fraction" and resuspend cell pellet. **TIP: Make sure there are no clumps or bubbles.**

23. Add an additional 1 mL of complete RPMI and mix thoroughly by gently pipetting up and down 15 times. **TIP: Make sure there are no clumps or bubbles.**

24. Aliquot 10 µL of the Pan Monocyte fraction into a Lo-Bind Microcentrifuge tube and proceed to cell count. **CRITICAL: See Appendix D1 for cell counting instructions.**

25. Move Pan Monocyte tube to incubator until Cell Stimulation (Chapter 5).

Chapter 5: Cell Stimulation

Materials Required:

- Complete RPMI (37°C)
- LPS 1 mg/mL (-20°C)
- 15 mL Centrifuge Tube (Pan Monocytes in complete RPMI, LPS Monocytes, Unstimulated Monocytes)
- T75 Flask, T25 Flask, 6 Well Plate, or 24 Well Plate
- Incubated Pan Monocyte Tube in complete RPMI
- 1 x Lo-Bind Microcentrifuge Tubes for Cell Count (label Pan Monocyte)

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 21°C.

Methods:

1. Thaw a vial of stock 1 mg/mL LPS at ambient temperature.

2. Vortex LPS for 5 seconds.

3. Prepare a working stock of LPS. Add 1 µL of 1 mg/mL LPS to 1 mL complete RPMI. Final concentration of LPS is 1 µg/mL. **CRITICAL: Working 1 µg/mL LPS stock should be made fresh. If there is any remaining 1 mg/mL LPS stock, discard and do not re-freeze.**

4. Vortex LPS working stock for 10 seconds. **TIP: Ensure contents are well-mixed.**

5. Take Pan Monocytes from the incubator from Chapter 4: Step 25.

6. Centrifuge Pan Monocytes for 10 minutes at 300 rcf.

7. After cells are centrifuged check for pellet.

8. Aspirate supernatant. **TIP: Be careful to not aspirate cell pellet.**

9. Resuspend Pan Monocyte cells in complete RPMI to a cell density of 1 x 105 cells/mL.

10. Split cells into two separate 15 mL centrifuge tubes. One labeled "LPS Monocytes" and the other "Unstimulated Monocytes".

11. Supplement the tube labeled "LPS Monocytes" with 10 ng/mL of LPS. **CRITICAL: Volume is dependent on number of cells.**

- Add 10 µL of 1 µg/mL LPS working stock for every mL of cell suspension in step 10. This yields a final concentration of LPS of 10 ng/mL.
- Use serological pipette to mix thoroughly. **CRITICAL: Be careful not to create bubbles. This will maximize even stimulation of cell suspension.**

12. Seed the monocyte suspension into the appropriately sized plate depending on the volume of the suspension (See Table 9).

Table 9: Plate and Volume for Monocyte Seeding

Plate	Volume
48 well plate	500 µL/well
24 well plate	1 mL/well
6 well plate	2 mL/well
T25 Flask	3-7 mL
T75 Flask	8-15 mL

13. Incubate plate for 24 hours at 37°C, 5% CO2

Chapter 6: Chip Thaw

Materials Required:

- IsoCode Chips in Vacuum Sealed Bag (-20°C)

Methods:

1. Take vacuum sealed bag containing IsoCode chips from -20°C. **CRITICAL: Chips must stay sealed until Chip Loading (Chapter 8).**

2. Place on bench to thaw at ambient temperature 30 - 60 minutes prior to use.

3. While chips and samples thaw, prepare liquid reagents and attach all reagent tubes to IsoLight. Refer to the IsoLight System Guide for detailed instructions.

Chapter 7: Cell Staining

Materials Required:

- LPS Stimulated Pan Monocyte Cells from Chapter 6
- 15 mL Centrifuge Tubes (LPS Monocyte and Unstimulated Monocyte)
- 1.5 mL Microcentrifuge Tube (Population Assay)
- Sterile 1X PBS (Room temperature)
- Complete RPMI (37°C)
- 1X Accutase (Room Temperature)
- Cell Scraper
- Mini-Cell Scraper
- Membrane Stain (-20°C)
- DMSO (-20°C)

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 21°C. Steps below describe parallel workflow for stimulated and unstimulated monocytes.

Methods:

1. Remove plate/flasks labeled "LPS Monocytes" and "Unstimulated Monocytes" from incubator.

2. Harvest monocytes in suspension by gently pipetting up the cell suspension.

- From "LPS Monocytes" plate/flask, add this cell suspension to "LPS Monocytes" tube.
- From "Unstimulated Monocytes" plate/flask, add this cell suspension to "Unstimulated Monocytes" tube.

3. Centrifuge cell suspension from Step 2 for 10 minutes at 300 rcf, to pellet non-adherent monocytes. This will ensure higher cell counts.

4. While cell suspension is centrifuging, rinse gently over the surface of the plate/flask with 1X PBS. Use 2 mL 1X PBS for a T25 flask, 5 mL for a T75 flask, 200 µL for a 48-well plate, 400 µL for a 24 well-plate, or 800 µL for a 6-well plate.

5. Tilt plate/flask and gently remove PBS from plate/flask.

- From plate/flask labeled "LPS Monocytes", remove PBS with a pipette and add to a tube labeled "PBS LPS Wash". **TIP: PBS rinse will contain monocytes. These will be combined in a later step to ensure higher cell counts.**
- From plate/flask labeled "Unstimulated Monocytes", remove PBS with a pipette and add to a tube labeled "PBS Unstimulated Wash". **TIP: PBS rinse will contain monocytes. These will be combined in a later step to ensure higher cell counts.**

6. Thaw 1X Accutase at room temperature. Pipet up and down for 5 times to ensure it is well mixed. Detach adherent cells from the plate/flask by gently adding 1X Accutase to all wells. Use 2 mL 1X Accutase for a T25 flask, 5 mL for a T75 flask, 200 µL for a 48-well plate, 400 µL for a 24 well-plate, or 800 µL for a 6-well plate.

7. Rock the plate/flask back and forth to ensure the 1X Accutase covers the entire bottom of the plate/flask.

8. Incubate the cells with 1X Accutase for 10 minutes at room temperature to allow the cells to detach. During incubation, be sure to rock the plate/flask back and forth once per minute. Observe the cells under the microscope until they appear detached.

9. After cells from step 3 are centrifuged, check for cell pellets.

10. Use a pipette and remove 1 mL of supernatant from each tube.

- Remove 1mL "LPS Monocytes" supernatant and add to microcentrifuge tube labeled "LPS Population Assay. Store at -80°C for population assay. Remaining cells/supernatant are used in step 11a.
- Remove 1mL "Unstimulated Monocytes" supernatant to microcentrifuge tube labeled "Unstimulated Population Assay." Store at -80°C for population assay. Remaining cells/supernatant are used in step 11b.

11. PBS wash from step 5 is combined with monocyte/supernatant remaining from step 10.

- Add "LPS PBS Wash" from step 5 (a) to the centrifuge tube labeled "LPS Monocytes" remaining from step 10a after aliquoting for population assay.
- Add "PBS Unstimulated Wash" from step 5 (b) to centrifuge tube labeled "Unstimulated Monocyte" remaining from step 10b after aliquoting for population assay.

12. After incubation with Accutase, rinse the plate/flask with complete RPMI to remove adherent monocytes.

- Gently rinse the plate/flask labeled "LPS Monocytes" with complete RPMI to remove LPS adherent fraction and add to the centrifuge tube labeled "LPS Monocytes". **TIP: Rinse gently, be careful not to create bubbles.**
- Gently rinse the plate/flask labeled "Unstimulated Monocytes" with complete RPMI to remove the unstimulated adherent fraction and add to the centrifuge tube labeled "Unstimulated Monocytes". **TIP: Rinse gently, be careful not to create bubbles.**

13. Use an appropriately sized cell scraper to remove additional monocytes from the bottom of each well, if needed. **TIP: Scrape the entire bottom of the well multiple times to ensure all monocytes are detached.**

CRITICAL: Observe wells under light microscope to ensure all cells were removed from the plate/flask. If not repeat step 13.

14. Centrifuge the tubes labeled “LPS Monocytes” and “Unstimulated Monocytes” for 10 min at 300 rcf.

15. After cells are centrifuged, check for cell pellets.

16. Aspirate supernatant. **TIP: Be careful not to aspirate the cell pellets.**

17. Add 1 mL of 1X PBS to dilute any remaining media and mix by pipetting up and down.

18. Centrifuge cells for 10 minutes at 300 rcf.

19. After cells are centrifuged, check for cell pellets.

20. Aspirate supernatant. **TIP: Be careful not to aspirate the cell pellets.**

21. Prepare membrane stain stock.

- Thaw tube of membrane stain diluent (DMSO) at room temperature.
- Spin tubes of membrane stain and membrane stain diluent (DMSO) in a mini centrifuge for 10 seconds to collect the contents at the bottom of the tubes.
- Add 20 µL of membrane stain diluent (DMSO) directly to the tube of membrane stain. Pipet up and down 15 times gently to resuspend.

CRITICAL: Membrane stain must be prepared fresh. Discard remaining stain—do not store.

22. Prepare stain master mix by diluting 2 µL of membrane stain into 1 mL of 1X PBS in a Lo-Bind microcentrifuge tube (1:500 final dilution). With the same pipette tip, pipette up and down 10 times to ensure all membrane stain has been released. Depending on sample number and cell count, additional tubes of stain master mix may need to be prepared. **CRITICAL: Failure to follow these steps will negatively impact cell counts.**

- With a P1000 set to 500 µL, gently pipette the stain master mix up and down 15 times.
- Gently vortex the stain master mix for 5 seconds.
- Ensure master mix is mixed well before adding stain to cells.

23. For every 1 x 106 cells, add 100 µL of stain master mix to each tube labeled “LPS Monocyte” and “Unstimulated Monocyte.” **CRITICAL: Pipet mix 15 times. Be careful not to create bubbles.**

24. Incubate for 5 minutes at 37°C in the dark.

25. Gently pipet, to mix the cell suspension, 15 times. **CRITICAL: Be careful not to create bubbles.**

26. Incubate for an additional 5 minutes at 37°C in the dark.

27. After incubation, add 5 times the volume of complete RPMI. **CRITICAL: Pipet mix 15 times gently. Be careful not to create bubbles.**

28. Incubate for 10 minutes at 37°C in the dark.

29. Take 10 µL of cells to count. Count cells using a hemocytometer and determine percent of viable cells as described in Appendix D1. **TIP: Cell counting can be done while cells are incubating.**

30. Centrifuge stained cells for 10 minutes at 300 rcf.

31. After cells are centrifuged, check for cell pellets.

32. Aspirate supernatant with a pipette. **TIP: Be careful not to aspirate the cell pellets.**

33. Resuspend the cells with complete RPMI to a cell density of 7.5 x 105 cells/mL. **CRITICAL: Pipet mix 30 times to reduce**

cell clumping. Be careful not to create bubbles. Proceed to the Chip Loading Chapter, as the cell suspension should be immediately pipetted into chips.

Chapter 8: Chip Loading

Materials Required (Pre-prepared):

- Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 6
- Stained Pan Monocyte Cells at 7.5 x 105 cells/mL

Methods:

1. Remove IsoCode chips from vacuum sealed bag and place on a flat surface. **CRITICAL: Keep protective blue film on bottom of chip.**

2. Resuspend "LPS Monocytes" and "Unstimulated Monocytes" tubes by gently pipetting up and down. Immediately proceed to chip loading. **CRITICAL: Pipet mix 30 times to reduce cell clumping. Be careful not to create bubbles.**

3. Pipette 40 µL of cell suspension into IsoCode chip. **CRITICAL: Be careful not to create bubbles. Insert pipette tip vertically into inlet port until tip lightly touches bottom, and slowly pipette 40 µL into inlet port. Be careful not to eject second step of pipette–it will cause bubbles.**

4. Let IsoCode chips sit for one minute on a flat surface.

5. Check bottom of chip to ensure liquid has entered the chip. **TIP: If liquid has not flowed, tap IsoCode chip on flat surface lightly.**

6. When inserting IsoCode chip into IsoLight, make sure barcode is facing up and towards you with the magnet facing the IsoLight. Take the blue film off while inserting each IsoCode chip into the IsoLight.

NOTE: Please refer to the loading instructions of the IsoLight instrument for details.

Notes and Comments

D: Appendix

D1 Protocol: Cell Quantification & Viability

Materials Required:

- Hemocytometer
- 10 µL aliquot of cells
- Trypan Blue

NOTE: Automated cell counters can be used in this protocol EXCEPT prior to loading cells on chip due to spectral overlap of the stains. Manual cell counting is required prior to loading on the chip.

1. Using a P10 pipette, add equal volume of Trypan blue solution to 10 µL of sample. Mix gently to resuspend. **TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.**

2. Load onto hemocytometer. **CRITICAL: Be careful not to overfill or create bubbles.**

3. Count and record viable (clear) and dead cells (blue) of all four 16-square corners.

CRITICAL: If more than 200 cells/16 squares were counted, repeat count using a 1:5 or 1:10 dilution with PBS or complete RPMI using a fresh sample aliquot.

4. Calculate the concentration of cells as follows:

- Concentration (cells/mL) = Average per square cell count x 104 x dilution factor

5. Calculate the number of cells as follows:

- Number of cells = Cell concentration (cells/mL) x total volume of cell suspension (mL)

6. Calculate percent viable cells:

- % Viable cells = 100 x number of viable cells / [number of viable cells + number of dead cells]

7. Calculate cell resuspension volume:

- Resuspension volume = Number of cells / Resuspension concentration
    - Example: 243,000 cells / 600,000 cells/mL = 0.405 mL = 405 µL

D2 Protocol: Dead Cell Removal Using Ficoll

Materials Required:

- Complete RPMI (37°C)
- Cells (Minimum 3x106)
- 2 x 15 mL Centrifuge Tubes
- Ficoll Paque

CRITICAL: It is recommended to start this protocol with a minimum of 3x106 total cells.

1. Carefully add 6 mL of Ficoll to the bottom of the required number of 15 mL centrifuge tube(s) prior to harvesting stimulation cultures.

2. Centrifuge cells for 10 minutes at 300 rcf.

3. Remove cells from centrifuge, check for cell pellet.

4. Aspirate supernatant. **TIP: Be careful not to aspirate cell pellet.**

- Use pipette to aspirate remaining supernatant.

5. Resuspend the pellet(s) in 7 mL of complete RPMI. **TIP: Be careful not to create bubbles.**

CRITICAL: Do not use more than 1 x 107 cells of your suspension per Ficoll tube.

6. Add the cell suspension(s) VERY SLOWLY to the tube(s) containing Ficoll. **CRITICAL: Place the tip of your pipette on the wall of the tube, close to the Ficoll layer. Add cell suspension VERY SLOWLY.**

CRITICAL: This step must be done carefully and slowly to avoid mixing of the layers.

7. Centrifuge tubes for 20 minutes at 300 rcf without brake or acceleration.

CRITICAL: Turn acceleration and brakes off to preserve the density layers established during centrifugation.

8. While cells centrifuge, prepare appropriate number of 15 mL centrifuge tube(s) containing 6 mL of complete RPMI.

9. Remove cells from centrifuge, check for cloudy layer which are the viable cells.

10. Aspirate a small volume of the supernatant. **CRITICAL: Be careful not to aspirate cloudy layer containing viable cells.**

11. Using a P1000 pipette, collect the viable cells by recovering the cloudy layer between Ficoll and complete RPMI media

12. Transfer cells into the 15 mL centrifuge tube(s) containing complete RPMI.

13. Aliquot 10 µL of cell/complete RPMI mixture(s) into a Lo-Bind Microcentrifuge Tube(s) and proceed to cell count. **CRITICAL: See Appendix D1 for cell counting instructions.**

For troubleshooting, see pages 23-24 on the PDF of the protocol, or contact Support at 475-221-8402 & support@isoplexis.com with specific troubleshooting questions.

References

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