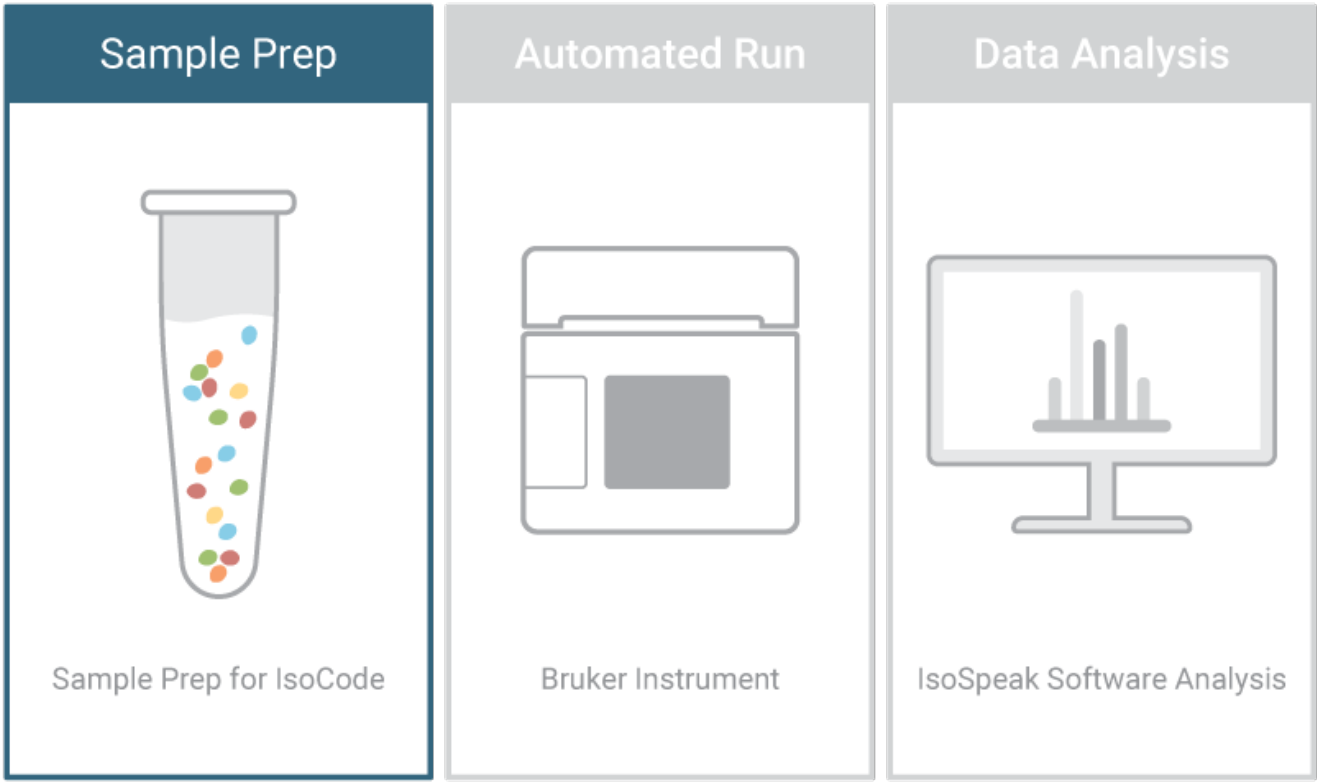
IsoCode Single-Cell Adaptive Immune: Human Monocyte-Derived Dendritic Cells Protocol

Ensure you achieve the maximum benefit from the Bruker systems and generate impactful data as quickly as possible



Key: ● TIP, ● CRITICAL, ● OPTIONAL

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A. Overview

Overview of Protocol

Day 1: Cryopreserved cells are thawed and immediately enriched for monocytes.

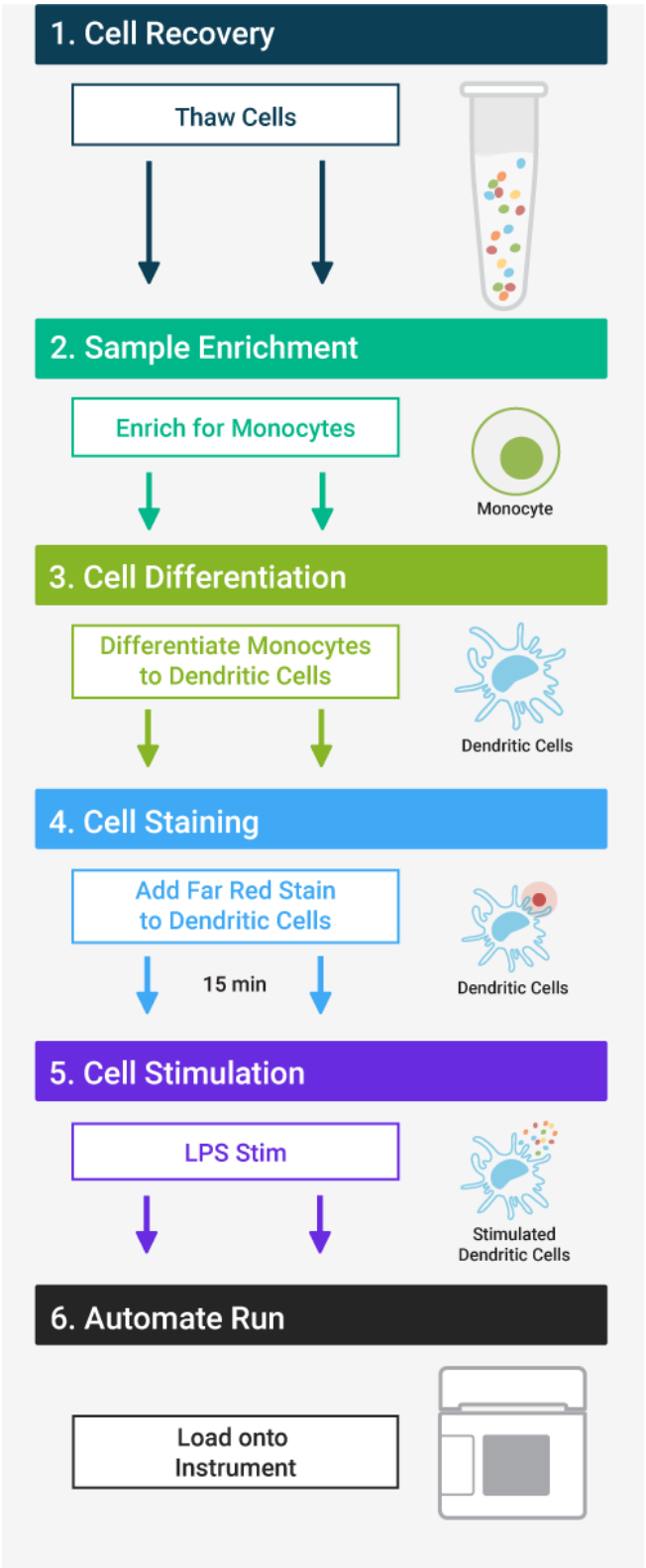
Day 4: Media is changed.

Day 6: Staining, stimulation, and loading of monocyte-derived dendritic cells onto IsoCode Chip.

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

NOTE:
For brevity, when this protocol refers to dendritic cells (DCs), this is meant to refer exclusively to human monocyte-derived dendritic cells.

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 Bruker will require validation prior to use. Please consider Bruker's IsoPACE program to assist in custom marker and protocol validation.



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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.

Required Reagents, Consumables and Equipment

Table 1: Required Consumables Provided by Bruker

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

IsoCode Kit Components

IsoLight 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

IsoSpark 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)
- Cartridge containing Reagents 1, 2, 3, and 4

IsoCode Chip Set (-20°C)

- Boxes of IsoCode Chips (2 per box)
  - IsoSpark: 4 chip kits
  - IsoLight: 4 or 8 chip kits

Table 2: Required Consumables Not Supplied by Bruker

Consumable	Type	Source	Catalog Number
T25 Flask	N/A	Corning	353108
T75 Flask	N/A	Corning	430641U
MACS LS Column	N/A	Miltenyi	130-042-401
Centrifuge Tubes*	Polypropylene, 15 mL	VWR	CA62406-200
Centrifuge Tubes*	50 mL	VWR	21008-242
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	USA Scientific	4043-1081
Cell scraper	N/A	Corning	3010
Fisherbrand Disposable PES Filter Units (0.20 µm)	500 mL	Fisher Scientific	FB12566504

*Bruker strongly recommends that low protein binding centrifuge tubes are used for cell culture work to ensure optimal cell pelleting.

Table 3: Required* Reagents Not Supplied by Bruker

Reagent	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	Gibco	10010072
GM-CSF	N/A	Peprotech	300-03
IL-4	N/A	Peprotech	200-04
RoboSep buffer	1x	StemCell Tech	20104
Miltenyi CD14 MicroBeads, Human	N/A	Miltenyi	130-050-201
Trypan Blue	0.4%	Gibco	15250-061
CellTrace Far Red Cell Proliferation Kit	N/A	Invitrogen	C34564
DMSO	N/A	Invitrogen	C34564
LPS (lyophilized powder)	N/A	Sigma	L2654-1MG
Reagent alcohol 70%	N/A	Lab Grade	N/A

*Reagents have been validated by Bruker and no alternatives may be used.

Table 4: Required Equipment

Equipment	Source	Catalog Number/Requirements
IsoLight, IsoSpark, or IsoSpark Duo Instrument	Bruker	ISOLIGHT-1000-1, ISOSPARK-1000-1, or ISOSPARK-1001-1
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 5: 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
Vortex Mixer	Ability to vortex vials and microcentrifuge tubes; adjustable speed

*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.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

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 6: LPS Recipe

- CRITICAL: LPS has been validated for use by Bruker. Using alternative stimulant may result in failed runs. Please contact your Field Application Scientist for additional information.

Ingredient	Stock Concentration	Final Concentration	Amount for 1 mL	Vendor/Catalog
LPS	N/A	1 mg/mL	1 mg	Sigma/L2654-1MG
Phosphate buffered saline (1XPBS) without Calcium or Magnesium	1x	1x	1 mL	Gibco/ 10010072

- CRITICAL: Prepare 10 µL 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 7: Complete RPMI Recipe

- CRITICAL: Complete RPMI media has been validated for use by Bruker. Using alternative media may result in failed runs. Please contact your Field Application Scientist for additional information.

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

Note | Sterile-filter through 0.20 µm filter before use. Store complete RPMI Media at 4°C and warm up to 37°C in water bath prior to use.

Additional Reagents to Be Prepared

NOTE: GM-CSF should be reconstituted in 1 mL of sterile deionized water upon receipt from vendor. Final concentration is 100 µg/mL. 10 µL aliquots are recommended and storage is at -20°C for up to 3 months.

NOTE: IL-4 should be reconstituted in 1 mL of sterile deionized water upon receipt from vendor. Final concentration is 50 µg/mL. 10 µL aliquots are recommended and storage is at -20°C for up to 3 months.

C. Protocol

Chapter 1: Getting Started

Kit Contents

IsoLight 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

IsoSpark 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)
- Cartridge containing Reagents 1, 2, 3, and 4

IsoCode Chip Set (-20°C)

- Boxes of IsoCode Chips (2 Per Box)
  - IsoSpark: 4 chip kits
  - IsoLight: 4 or 8 chip kits

Chapter 2: Thawing of Cryopreserved Cells

Materials Required

Complete RPMI (37°C)
Cryopreserved PBMC
15 mL Centrifuge Tube
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. 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 media when pipetting, be careful to not create bubbles.
- 6. Take 1 mL of complete RPMI and 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.
- 7. Draw up cell/complete RPMI mixture and pipette into the 15 mL centrifuge tube, labeled *Thawed PBMC*. TIP: Insert tip into complete RPMI and pipette gently up and down. Be careful to not create bubbles.
- 8. Mix well 5 times with 10 mL serological pipette. TIP: Be careful not to create bubbles.
- 9. 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.
- 10. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.

11. Proceed immediately to next chapter.

Chapter 3: CD14 Sample Enrichment and Differentiation

Materials Required

Complete RPMI (37°C)
GM-CSF 100 µg/mL (-20°C)
IL-4 50 µg/mL (-20°C)
15 or 50 mL Centrifuge Tube (*Differentiation Media*)
T75 Flask(s) or T25 Flask(s)
RoboSep Buffer (4°C)
Miltenyi CD14 Microbeads, Human, 2 mL (4°C)
MACS LS Column
Prepared Cells from Chapter 2
Enrichment Kit:
    MACS Metal Plate/Magnet Kit
    3 x 15 mL Centrifuge Tubes (*Discard, Flow Through, CD14 fraction*)
    Lo-Bind Microcentrifuge Tube for Post-Enrichment CD14

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.
    a. Use pipette to aspirate remaining supernatant.

- CRITICAL: For every 1 x 10^7 cells, resuspend in 80 µL RoboSep (4°C) and 20 µL of CD14 beads (4°C).

3. Add 80 µL of cold RoboSep to 15 mL centrifuge tube containing 1 x 10^7 or fewer cells.
4. Vortex the Miltenyi CD14 Microbeads at a slow speed for 10 seconds.
5. Add 20 µL of Miltenyi CD14 Microbeads and mix well by gently pipetting up and down 5 times.

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

- 6. Incubate in refrigerator (4°C) for 15 minutes. TIP: Don't incubate on ice as increased incubation times may be required.
- 7. After 15 minutes, add 2 mL of cold RoboSep. TIP: Not necessary to mix for this step.

8. Centrifuge cells for 10 minutes at 300 rcf.

- TIP: Keep RoboSep in refrigerator during enrichment process.

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

10. After cells are centrifuged, check for cell pellet and continue with MACS separation.

- 11. Aspirate RoboSep from cell pellet. TIP: Since it is a small volume, use pipette for this step to prevent accidental aspiration of the cell pellet.

12. For 1 x 10^8 or fewer cells, resuspend with 500 µL of cold RoboSep.

- a. Mix well to resuspend by gently pipetting up and down 5 times. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.

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

- 13. Start with the LS column over the "Discard" 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.

14. Unscrew and keep cap for "Flow Through" tube. NOTE: This is in preparation for next step to ensure the column does not dry out during the transition.

- 15. When the last drop falls through to the "Discard" tube, move the rack over so the LS column is over the "Flow Through" 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.

16. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.
17. Mix cell suspension by gently pipetting up and down 5 times. NOTE: This ensures that the cells are evenly dispersed after sitting.
18. Draw up all 500 µL of cell suspension and pipette carefully into the center of the LS column without touching sides of the column.
19. Wash 3 times with 3 mL of cold RoboSep.

a. 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.

i. Pipette all the mixture into LS Column after last drop passes through or does not fall from step 18. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.

b. 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.

c. 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.

20. After the last drop of the third wash passes through or does not fall, remove the LS Column carefully from the magnet, and place carefully on the tube labeled for "CD14 fraction."
21. Cap the "Flow Through" tube. Keep if performing further enrichments. Otherwise, discard.
22. Add 5 mL of cold RoboSep to the LS column. CRITICAL: Be careful not to touch the sides.
23. Take plunger, smoothly push down on the plunger to push the RoboSep buffer through the LS Column. TIP: Lift up at the end of the plunging action so that the liquid does not splash back onto the LS Column tip.
24. Set LS Column back on the "CD14 fraction" tube.

CRITICAL: Do not allow the plunger to interact with external contaminants. It will be used for one more step.

25. Loosen up plunger. Remove plunger briefly from column and hold in one hand.
26. Add another 4 mL of cold RoboSep to the LS Column.
27. Take plunger, smoothly push down on the plunger to push the RoboSep buffer through the LS Column. TIP: Lift up at the end of the plunging action so that the liquid does not splash back onto the LS Column tip.
28. Discard LS Column and plunger.
29. Aliquot 10 µL of the "CD14 fraction" into a Lo-Bind microcentrifuge tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
30. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.
31. Based on cell number obtained and Table 8, determine appropriately sized flask and required volume of media. NOTE: If needed, additional flasks could be used in a manner following the parameters of the table.

Table 8: Flask and Volume for CD14 Fraction Seeding

Flask	Cell Density (cells / mL)	Minimum Vol. in Flask (mL)	Maximum Vol. in Flask (mL)	Cell Number Range (cells)
T25 Flask	5×10^5	3	10	$1.5 \times 10^6 – 5 \times 10^6$
T75 Flask	5×10^5	10	25	$5 \times 10^6 – 1.25 \times 10^7$

32. Prepare differentiation media, complete RPMI with GM-CSF and IL-4, in a centrifuge tube.
    a. For every 5×10^5 cells, aliquot 1 mL of complete RPMI in 15 mL or 50 mL centrifuge tube.
    b. Add 1 µL of GM-CSF (100 µg/mL) per mL of complete RPMI aliquoted in step 32a. Final concentration is 100 ng/mL.
    c. Add 1 µL of IL-4 (50 µg/mL) per mL of complete RPMI aliquoted in step 32a (w/GM-CSF). Final concentration is 50 ng/mL.
    d. Use serological pipette to mix thoroughly.
33. Add ~80% of prepared differentiation media to flask. NOTE: The flask does not contain cells in this step. If using multiple flasks, the differentiation media should be split equally across the flasks.
34. Remove the centrifuged cells and check for cell pellet.
35. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
36. Resuspend cell pellet in 1 mL of the remaining differentiation media using a P1000. TIP: Make sure there are no clumps or bubbles.
37. Add any of the remaining differentiation media, originally prepared in step 32, to the 15 mL centrifuge tube containing the CD14 cell suspension. Mix thoroughly by gently pipetting up and down 5 times. TIP: Make sure there are no clumps or bubbles.
38. Seed the CD14 cell suspension into the prepared flask(s) from step 33. NOTE: If using multiple flasks, the cell suspension should be split equally across the flasks.
39. Spread out cell suspension by gently rocking the flask back and forth.
40. Incubate flask(s) for 4 days at 37°C, 5% CO_2.

Chapter 4: Media Change

Materials Required

Complete RPMI (37°C)
GM-CSF (-20°C)
IL-4 (-20°C)
15 mL Centrifuge Tube (*Differentiation Media*)

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

Methods

NOTE: If working with multiple flasks, conduct the following steps in Chapter 4 for each flask in parallel.

1. Take cells out of the incubator. CRITICAL: Do not remove cells from incubator prior to day 4.
2. Observe flask under light microscope, using a 10X objective, to ensure cells are exhibiting dendritic-like morphology. NOTE: Most cells are in suspension. Some cells will adhere to the flask. Differentiated dendritic cells are globular and lack uniformity of shape. Some cells may exhibit small projections.
3. Transfer one half of the cell suspension from flask into 15 mL centrifuge tube. NOTE: Only one half of the cell suspension is removed to ensure any adherent cells remain in differentiation media.
4. Centrifuge cells for 10 minutes at 300 rcf.
5. While cells are centrifuging, prepare differentiation media, complete RPMI with GM-CSF and IL-4, in a centrifuge tube.
    a. Aliquot the same volume of complete RPMI, in a 15 mL centrifuge tube, that was removed from the flask in step 3.
    b. Add 2 µL of GM-CSF (100 µg/mL) per mL of complete RPMI aliquoted in step 5a. Final concentration is 200 ng/mL. NOTE: Final concentration is 2x. When fresh media is added to the other half of the cell suspension remaining in the flask it will result in 1x differentiation media.
    c. Add 2 µL of IL-4 (50 µg/mL) per mL of complete RPMI aliquoted in step 5a (w/GM-CSF). Final concentration is 100 ng/mL. NOTE: Final concentration is 2x. When fresh media is added to the other half of the cell suspension remaining in the flask it will result in 1x differentiation media.
    d. Use serological pipette to mix thoroughly.
6. Remove the centrifuged cells and check for cell pellet.
7. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
8. Resuspend cell pellet in 1 mL of the differentiation media, prepared in step 5, using a P1000. TIP: Make sure there are no clumps or bubbles.
9. Add the remaining differentiation media to the cell suspension. Mix thoroughly by gently pipetting up and down 5 times. TIP: Make sure there are no clumps or bubbles.
10. Transfer cell/differentiation media mixture to flask.
11. Spread out cell suspension by gently rocking the flask back and forth.

12. Incubate flask for an additional 2 days at 37°C, 5% CO_2. NOTE: Cells will continue to differentiate and are expected to look similar between day 4 and day 6.

Chapter 5: Chip Thaw

Materials Required

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

Methods

NOTE: Complete Chapter 5 immediately before starting Chapter 6.

1. Take vacuum sealed bag containing IsoCode chips from -20°C. CRITICAL: Chips must stay sealed until Chip Loading (Chapter 7).
2. Place on bench to thaw at ambient temperature 30 - 60 minutes prior to use.
3. While chips thaw, prepare liquid reagents and setup in the Bruker instrument. Refer to your instrument's system guide for detailed instructions.

Chapter 6: Cell Staining and Stimulation

Materials Required

Differentiated Dendritic Cells in Flask(s) in Differentiation Media from Chapter 4
50 mL Centrifuge Tube
15 mL Centrifuge Tubes (*Stain Master Mix, Unstimulated DCs, Stimulated DCs, LPS Complete RPMI, Complete RPMI Unstimulated)*
Lo-Bind Microcentrifuge Tubes (*Stain Working Stock, Cell Count)*
Sterile 1X PBS (Room Temperature)
Complete RPMI (37°C)
Cell Scraper
LPS 1 mg/mL (-20°C)
CellTrace Far Red (-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.

Methods

1. Take dendritic cells out of the incubator.
2. Observe flask(s) under light microscope, using a 10X or 20X objective, to ensure cells are exhibiting dendritic-like morphology.

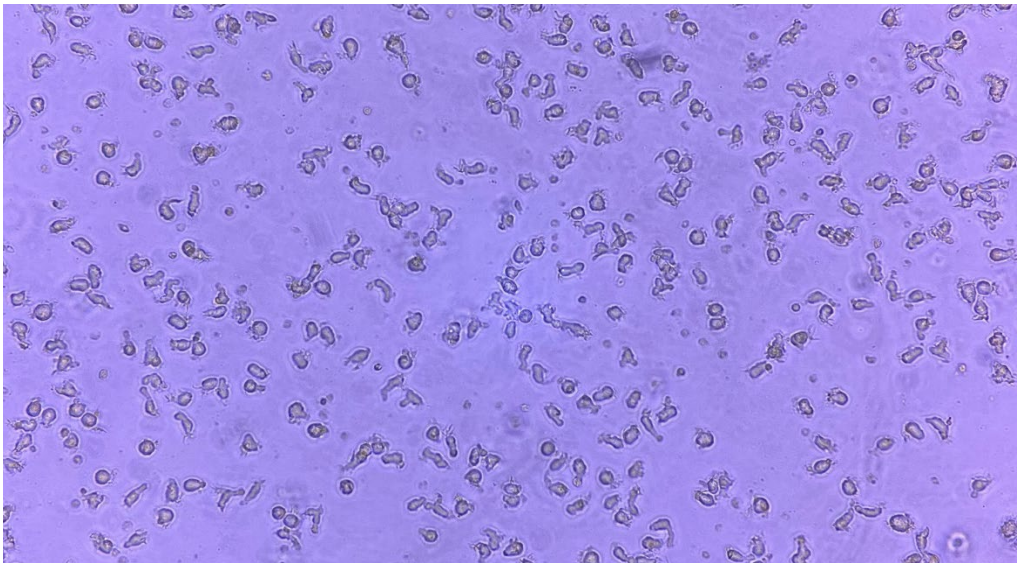

Figure 1. Example cell morphology at day 6 using a 20X objective.

NOTE: Differentiated dendritic cells are globular and lack uniformity of shape. Some cells may exhibit small projections. Some dead cells are expected after differentiation. Any small dark circles observed are cells that did not survive the differentiation. Dead cells will not pellet in later steps and will be removed in the centrifugation process.

3. Add complete RPMI to flask and rinse 5 times. TIP: Make sure to spread out the complete RPMI to gather maximum number of cells.
    - For T75 Flask add 3 mL
    - For T25 Flask add 2 mL
4. Transfer cell/complete RPMI mixture to 50 mL centrifuge tube.
5. Gently rinse the flask with 1X PBS.
    - For T75 Flask add 5 mL
    - For T25 Flask add 2 mL
6. Observe the flask under the microscope to determine if any dendritic cells may have adhered to the flask during the differentiation process.
    a. If needed, gently use a cell scraper to dislodge adhered dendritic cells. After scraping, gently rinse over the surface of the flask with the 1X PBS in the flask.
7. Collect cells/1X PBS mixture and transfer to 50 mL centrifuge tube containing the dendritic cell suspension from step 4.
8. Centrifuge cells for 10 minutes at 300 rcf.
9. After cells are centrifuged, check for cell pellet.
10. Remove the majority of the supernatant with a P1000 or P200 pipette. Switch to a P10 pipette to remove all media from the cell pellet. TIP: Be careful not to aspirate the cell pellet.

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

- CRITICAL: Failure to remove excess media will result in poor staining.

- 12. Mix well 5 times with 10 mL serological pipette. TIP: Be careful not to create bubbles.
- 13. 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.
- 14. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.

15. While cells are centrifuging, prepare CellTrace Far Red stock.
    a. Thaw tube of DMSO at room temperature.
    b. Spin tubes of CellTrace Far Red and DMSO in a mini centrifuge for 10 seconds to collect the contents at the bottom of the tubes.
    c. Add 20 µL of DMSO directly to the tube of CellTrace Far Red. Pipet up and down 15 times gently to resuspend.

- CRITICAL: CellTrace Far Red must be prepared fresh. Discard remaining stain—do not store.

NOTE: DMSO tube is stored at -20°C after preparing the CellTrace Far Red Stock.

16. Prepare a working stock by diluting 2 µL of CellTrace Far Red into 398 µL of 1X PBS in a 1.5 mL Lo-Bind microcentrifuge tube (1:200 dilution). With the same pipette tip, pipette up and down 10 times to ensure all CellTrace Far Red has been released.
    a. With a P100 set to 50 µL, gently pipette the stain working stock up and down **15 times**.
    b. **Gently vortex** the stain working stock for **5 seconds**.
    c. **Ensure working stock is mixed well.**

- 17. Prepare stain master mix by diluting 100 µL of CellTrace Far Red working stock into 10 mL of 1X PBS in a 15 mL centrifuge tube (1:100 dilution of working stock, 1:20,000 final dilution). With the same pipette tip, pipette up and down 10 times to ensure all CellTrace Far Red 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.
    a. With a 5 mL serological pipette, gently pipette the stain master mix up and down **15 times**.
    b. **Gently vortex** the stain master mix for **5 seconds**.
    c. **Ensure master mix is mixed well before adding stain to cells.**

18. After cells are centrifuged, check for cell pellet.
19. Remove the majority of the supernatant with a P1000 or P200 pipette. Switch to a P10 pipette to remove all media from the cell pellet. TIP: Be careful not to aspirate the cell pellet.
20. Gently remix stain master mix.

- CRITICAL: Failure to remix stain master mix will result in poor staining.

- 21. For every 1 x 10^6 cells, add 1 mL of well mixed stain master mix to the cell suspension tube. CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.
22. Incubate for 7.5 minutes at 37°C in the dark.
- 23. Gently pipet to mix the cell suspension 15 times. CRITICAL: Be careful to not create bubbles.
24. Incubate for an additional 7.5 minutes at 37°C in the dark.
- 25. After incubation, add the same volume of complete RPMI. CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.
26. Take 10 µL of cells to count. Count cells using a hemocytometer and determine percent of viable cells as described in Appendix D1.
27. Split the cell suspension in half in preparation for an unstimulated and stimulated condition.
    a. Pipette one half of the cell suspension into a 15 mL centrifuge tube labeled "Unstimulated DCs".
    b. Pipette the other half of the cell suspension into a 15 mL centrifuge tube labeled "Stimulated DCs".
28. Centrifuge "Unstimulated DCs" and "Stimulated DCs" for 10 minutes at 300 rcf.
29. While cells are centrifuging, thaw a vial of stock 1 mg/mL LPS at ambient temperature.
30. Vortex LPS for 5 seconds.
- 31. Prepare LPS complete RPMI mixture. Aliquot 10 mL complete RPMI into a centrifuge tube labeled "LPS Complete RPMI". CRITICAL: Volume required is dependent on number of cells.
    a. Aliquot 10 mL complete RPMI into a 15 mL centrifuge tube labeled "LPS Complete RPMI."
    b. Add 10 µL of LPS (1 mg/mL) to the complete RPMI. Final concentration is 1 µg/mL.
    c. Use serological pipette to mix thoroughly.
- CRITICAL: Discard remaining/unused LPS—aliquots are single use only and cannot be refrozen.
32. Prepare complete RPMI Unstimulated media.
    a. Aliquot 10 mL complete RPMI into a 15 mL Centrifuge Tube labeled "Complete RPMI Unstimulated" —set this complete RPMI aside as it will serve as the complete RPMI used for the unstimulated (negative control) condition.
33. After cells are centrifuged, check for cell pellet.
- 34. Aspirate supernatant with pipette. TIP: Be careful not to aspirate the cell pellet.
35. Using the "Complete RPMI Unstimulated" media, resuspend the cells in the 15 mL centrifuge tube labeled "Unstimulated DCs" to a cell density of 1 x 10^6 cells/mL.
36. Use a pipette to mix the "LPS Complete RPMI" to ensure it is evenly distributed.
37. Using the "LPS Complete RPMI" from step 36, resuspend the cells in the 15 mL centrifuge tube labeled "Stimulated DCs" to a cell density of 1 x 10^6 cells/mL.
38. Procced immediately to Chapter 7.

Chapter 7: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 5 Stained Stimulated and Unstimulated Dendritic Cells at 1 x 10^6 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 "Stimulated DCs" and "Unstimulated DCs" by gently pipetting up and down. Immediately proceed to chip loading. CRITICAL: Pipet to mix 30 times to reduce cell clumping. Be careful not to create bubbles.
3. Pipette 30 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 30 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 instrument, make sure the logo is facing up and towards you with the magnet facing the instrument. Take the blue film off while inserting each IsoCode chip into the instrument.

   NOTE: Please refer to your instrument's loading instructions for details.

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.

NOTE: To obtain an accurate representation of cell viability, cells should be counted within 15 minutes of staining as cell viability will drop over time because Trypan Blue is toxic.

1. Quick spin the Trypan Blue to pellet potential debris. Remove aliquot from the top of Trypan Blue.
2. 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.
3. Load onto hemocytometer. CRITICAL: Be careful not to overfill or create bubbles.
4. 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.

5. Calculate the concentration of cells as follows:
   a. Concentration (cells/mL) = Average per square cell count x 10^4 x dilution factor
6. Calculate the number of cells as follows:
   a. Number of cells = Cell concentration (cells/mL) from D.1.5 x total volume of cell suspension (mL)
7. Calculate percent viable cells:
   a. % Viable cells = 100 x number of viable cells / [number of viable cells + number of dead cells]

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Troubleshooting & References

Please contact Support at 844-476-7539 (toll free) or 475-221-8402 or email support@isoplexis.com with specific troubleshooting questions.

Problem	Possible Reason	Solution
Low quality cell count on chip *Cell Counting & Concentration related*	• Recommended cell concentrations not used • Issue with Cell Counting procedure • Trypan Blue may have debris • Trypan Blue is toxic • Poor cell removal from plate	• Use recommended cell concentrations during incubation (**Chapter 6**) • Use appropriate dilutions recommended in **Appendix D1**. • Do a recount if initial count does not seem accurate • Quick spin Trypan Blue to pellet potential debris, remove aliquot from top of Trypan Blue. Start with fresh aliquot of Trypan Blue. • Count within 15 minutes of staining cells • Thoroughly rinse cells in flask with pipette prior to transferring to tube (**Chapter 6**)
Low quality cell count on chip *Stain Process related*	• Use of media other than the recommended media in protocol which could interact with stain • Use of stains not recommended in protocol • Recommended stain concentration, incubation time and/or incubation temperature not used • CellTrace Far Red not stored at -20°C prior to use • Media not completely removed from cell pellet prior to staining	• Use complete RPMI media following recipe in **Table 7** • Use Bruker validated stain • Follow staining steps as highlighted in **Chapter 6.** • Use only freshly prepared CellTrace Far Red per **Chapter 6** • Ensure all media is removed from cell pellet in **step 6.10**

Low quality cell count on chip *Technique Detail related*	• Bubbles loaded onto chip, especially at Chip Loading
	• Detection of potential artifacts such as debris, cell clumping, inefficient enrichment possibly due to:
	  ○ Pipetting wrong concentration
	  ○ Not fully inserting column into MACS separator
	  ○ Reagents not stored at recommended temperatures
	• Recommended number of cells not loaded on chip
	• Cell pellet or cells lost during centrifuging
		• Follow Critical steps **7.2** and **7.3** to avoid introduction of bubbles on chip
		• Ensure use of a sterile space to reduce introduction of potential contaminants. Use dedicated pipettes, tips, and tubes for sterile work. Pipette up and down gently and throughout protocol to reduce clumps.
		• Follow closely the Critical steps and tips in **Chapter 3** (CD14 Sample Enrichment)
		• Load recommended number of cells (30,000 cells per chip) (**Chapter 7**)
		• Use low protein binding centrifuge tubes

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Viability related*	• Leaving thawed cells in DMSO for an extended period • Decreased viability due to cell shock	• After thaw, quickly transfer cells from DMSO to complete RPMI to ensure viability of cells. • Use reagents at recommended temperatures (i.e. always use warmed media [37°C])
Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Stimulation step related*	• Recommended LPS stimulation concentration was not used • Expired LPS used	• Use LPS concentration listed in **Chapter 6** • Do not use LPS stock older than 2 months • Use required vendors as listed in **Table 3**