IsoCode Single-Cell Innate Immune: Human CD34+ Myeloid Stem Cell Protocol

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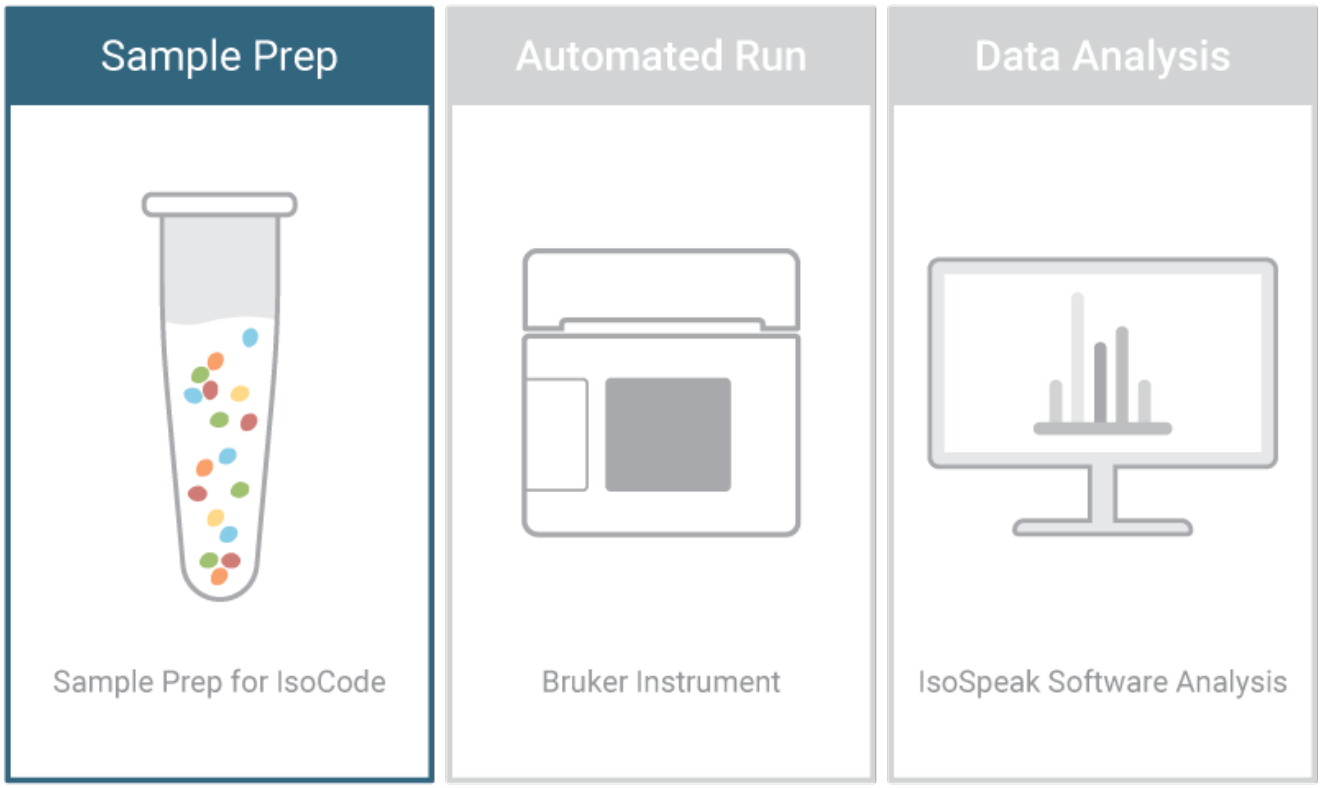

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Contents

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A. Overview

Overview of Protocol

Day 1: Cryopreserved cells are thawed and cultured overnight in the presence of IL-2.

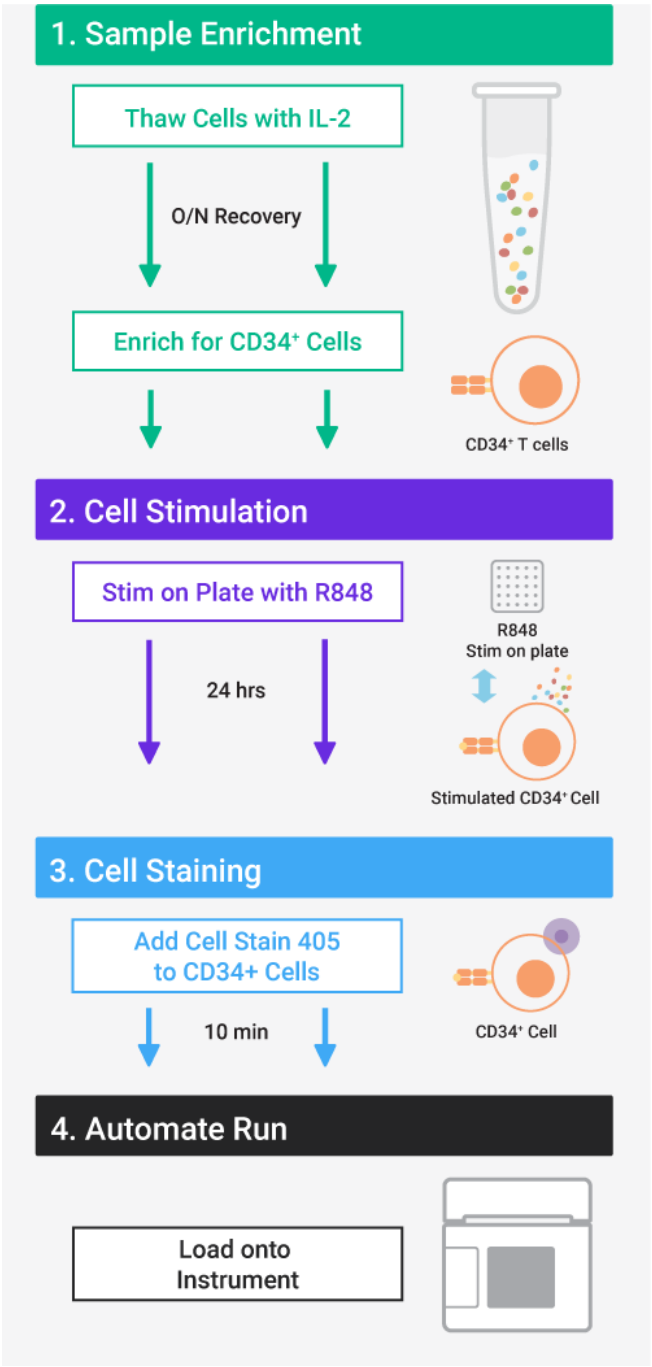
Day 2: Enrichment and Stimulation of CD34+ cells for 24 hours.

Day3: Staining and Loading of CD34+ cells onto IsoCode chip.

NOTE:
This protocol outlines the standard method for thawing and culturing of human CD34+ myeloid stem cells (such as from AML Blasts) and may not necessarily be valid for other species or CD34+ labeled cells from other tissues (such as endothelial progenitor cells).

NOTE:
For brevity, when this protocol refers to CD34+ cells, this is meant to refer exclusively to human CD34+ myeloid stem 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.



Safety Warnings

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- 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 Reagents and 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
- Cell Stain 405 [ordered separately]
- Cell Stain 405 Diluent (DMSO) [part of cell stain 405 kit]

Table 2: Required Consumables Not Supplied by Bruker

Consumable	Type	Source	Catalog Number

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T25 Flask	N/A	Corning	353108
T75 Flask	N/A	Corning	430641U
6 Well Plate Flat-Bottom	N/A	Corning	353046
96 Well Plate Flat-Bottom	N/A	Corning	353072
MACS LS Column	N/A	Miltenyi	130-042-401
Centrifuge Tubes*	Polypropylene, 15 mL	VWR	CA62406-200
Lo-Bind Microcentrifuge Tubes, Sterile	1.5 mL	USA Scientific	4043-1081
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	2 mL Pipette 5 mL Pipette 10 mL Pipette	USA Scientific	1072-0510 1075-0110 1071-0810
Syringe with BD Luer-Lok Tip	10 mL	VWR	75846-756
0.2 µm Syringe Filter with Acrylic Housing	N/A	VWR	28145-501
Fisherbrand Disposable PES Filter Units (0.20 µm)	500 mL	Fisher Scientific	FB12566504
Cell scraper	N/A	Corning	3010
Mini cell scraper	N/A	Biotium	22003

*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

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RPMI	1x	Fisher	MT10040CV
Penicillin-Streptomycin-Neomycin Solution Stabilized	100x	Sigma	P4083-100mL
Glutamax	100x	Thermo	35050061
FBS	1x	Sigma	F2442-6X500mL
Recombinant IL-2	200 µg/mL	Biolegend	589104
Bovine Serum Albumin (BSA), lyophilized powder	N/A	Sigma-Aldrich	A9647-10G
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	Gibco	10010072
Robosep buffer	1x	StemCell Tech	20104
Ficoll Paque Plus	N/A	GE Healthcare	17-1440-03
CD34 MicroBead Kit UltraPure, human, 2 mL	N/A	Miltenyi	130-100-453
Trypan Blue	0.4%	Gibco	15250-061
R848 (Resiquimod, lyophilized)	N/A	InvivoGen	tlrl-r848
Endotoxin-free Water	1x	InvivoGen	tlrl-r848
Peripheral Blood Mononuclear Cells (PBMCs) or Bone Marrow Mononuclear Cells (BMNCs)		Experiment Specific	
Reagent alcohol 70%	N/A	Lab Grade	N/A

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

Table 4: Cell Staining Reagents

Test Material	Catalog Number	Color

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Cell Stain 405	STAIN-1001-1	Violet

Table 5: Required Equipment

Equipment	Source	Catalog Number
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 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 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.

B. Before Getting Started

1. Important Precautions

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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 should 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: 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.

Table 8: 1% BSA Recipe

Ingredient	Stock Concentration	Final Concentration	Amount for 100 mL	Vendor/Catalog

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Bovine Serum Albumin (BSA), lyophilized powder	N/A	1%	1 g	Sigma-Aldrich/ A9647-10G
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1X	1X	99 mL initially*	Gibco/10010072

*Rotate solution until BSA powder is dissolved and then bring final volume up to 100 mL with 1X PBS.

Table 9: Working Stock of Recombinant IL-2 (1 µg/mL) Recipe

Ingredient	Stock Concentration	Final Concentration	Amount for 10 mL	Vendor/Catalog
1% BSA in 1X PBS (sterile filtered)	1%	1%	9.95 mL	Table
Recombinant IL-2	200 µg/mL	1 µg/mL	50 µL	BioLegend/589104

Note | Sterile-filter through a 0.2 µm PES filter before use.

- CRITICAL: Prepare 200 µL IL-2 aliquots and freeze at -20°C for no longer than 1 month. 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 10: Working Stock of R848 (1 mg/mL) Recipe

Ingredient	Stock Concentration	Final Concentration	Amount for 500 µL*	Vendor/Catalog
Endotoxin-free Water	1x	1x	500 µL	Invivogen/tlrl-r848
R848	N/A	1 mg/mL	500 µg	Invivogen/tlrl-r848

*Vortex solution until R848 is completely dissolved.

- CRITICAL: Prepare 15 µL R848 aliquots and freeze at -20°C for no longer than 6 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.

C. Protocol

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Chapter 1: Getting Started

Kit Contents

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
- Cell Stain 405 [ordered separately]
- Cell Stain 405 Diluent (DMSO) [part of cell stain 405 kit]

Chapter 2: Recovery of Cryopreserved Cells

Materials Required

Complete RPMI (37°C)
Recombinant IL-2 at 1 µg/mL (-20°C)
Cryopreserved PBMC or BMNC
15 mL Centrifuge Tube
Plate and/or Flask
- For > 10 M cells, T75 Flask
- For 6 - 9.9 M cells, T25 Flask
- For < 6 M, 6 Well Plate

All the following steps should take place in a sterile tissue culture hood.

Methods

1. Pipette 5 mL of 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.

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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 under 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 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 back 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. Centrifuge cells for 10 minutes at 300 rcf.
9. While the cells are centrifuging, take the IL-2 (1 µg/mL) out from -20°C and thaw at room temperature.

CRITICAL: Use IL-2 aliquot that has been frozen at -20°C for less than a month. Do not use IL-2 that has been previously thawed.

10. After cells are centrifuged, check for cell pellet.
11. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to remove last bit of supernatant.
12. Resuspend cell pellet in 1 mL of fresh complete RPMI.
    a. 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 10^6 cells/mL.
14. Mix thawed IL-2 thoroughly by carefully pipetting up and down.
15. Dilute 100 µL of 1 µg/mL IL-2 per 10 mL of cell suspension to a final concentration of 10 ng/mL IL-2.

CRITICAL: Discard thawed IL-2 aliquot if there is any volume remaining. IL-2 must only be thawed once.

16. Mix with serological pipette. TIP: Gently pipet up and down 3-5 times, be careful to not create bubbles.
17. Transfer cell suspension to flask or plate. TIP: Slowly pipette down the side of the flask as to not create bubbles.
18. Spread out cell suspension by rocking flask or plate carefully to fully cover the bottom of the container. TIP: Be careful to not make bubbles.
19. Move to incubator for overnight recovery at 37°C, 5% CO_2. NOTE: The time period for overnight recovery is considered 16 – 20 hours, but not exceeding 24 hours.

Chapter 3: Post-Recovery Sample Setup

Materials Required

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Complete RPMI (37°C)
15 mL Centrifuge Tube
Overnight Recovered Cells from Chapter 2 or Fresh Samples
Lo-Bind Microcentrifuge Tube for Cell Count
Cell Scraper or Mini Cell Scraper

All the following steps should take place in a sterile tissue culture hood.

Methods

1. Transfer suspension cells from flask or plate into 15 mL centrifuge tube.
2. Add complete RPMI to flask or plate and rinse 5 times. If necessary, use a cell scraper or mini cell scraper to remove adherent cells for combining into the same 15 mL centrifuge tube. TIP: Make sure to spread out the complete RPMI to gather maximum number of cells.
    - For T75 Flask: 3 mL
    - For T25 Flask: 2 mL
    - For 6 Well Plate: 1 mL
3. Transfer cell/complete RPMI mixture to the 15 mL centrifuge tube.
4. Mix well 5 times with 10 mL serological pipette. TIP: Be careful not to create bubbles.
5. 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.
6. 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.
7. CRITICAL: If cells are less than 80% viable, proceed to Appendix D2 Dead Cell Depletion Protocol using Ficoll.
    - Alternatively, if cell counts are extremely low, perform a low speed spin. Please refer to "Protocol: Dead Cell Removal Using Low Speed Spin" found in Appendix D3 of this protocol.
8. Proceed immediately to next chapter.

Chapter 4: CD34 Sample Enrichment

Materials Required

Complete RPMI (37°C)

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RoboSep Buffer (4°C)
Miltenyi CD34 Microbeads Kit, UltraPure, Human (4°C):
    CD34 Microbeads UltraPure, Human
    FcR Blocking Reagent, Human
MACS LS Column
Prepared Cells from Chapter 3
Enrichment Kit:
    MACS Metal Plate/Magnet Kit
    3 x 15 mL Centrifuge Tubes (*Discard, Flow Through, CD34 fraction*)
    Lo-Bind Microcentrifuge Tube for Post-Enrichment CD34

All the following steps should take place in a sterile tissue culture hood.

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 10^8 cells, resuspend in 300 µL RoboSep (4°C), 100 µL of CD34 beads (4°C), and 100 µL of FcR Blocking Reagent (4°C).

    e.g. for 2 x 10^8 cells, use 600 µL RoboSep (4°C), 200 µL of CD34 beads (4°C), and 200 µL of FcR Blocking Reagent (4°C).

3. Add 300 µL of cold RoboSep to 15 mL centrifuge tube containing 1 x 10^8 or fewer cells.
4. Vortex the Miltenyi FcR Blocking Reagent at a slow speed for 10 seconds.
5. Add 100 µL of Miltenyi FcR Blocking Reagent 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. Vortex the Miltenyi CD34 Microbeads UltraPure at a slow speed for 10 seconds.
7. Add 100 µL of CD34 Microbeads UltraPure 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.

- 8. Incubate in refrigerator (4°C) for 30 minutes. TIP: Don't incubate on ice as increased incubation times may be required.
- 9. After 30 minutes, add 7 mL of cold RoboSep for 1 x 10^8 or fewer cells. TIP: Not necessary to mix for this step.

10. Centrifuge cells for 10 minutes at 300 rcf.

- TIP: Keep RoboSep in refrigerator during enrichment process.

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11. 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.
12. After cells are centrifuged, check for cell pellet and continue with MACS separation.
13. 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.
14. 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.

    TIP: For higher cell counts, scale buffer volume, i.e. 2 x 10^8 resuspend with 1 mL of cold RoboSep.

    CRITICAL: Be careful not to let column dry out. Do not add liquid when there is already liquid in the LS Column.
15. 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.
16. 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.
17. 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.
18. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.
19. Mix cell suspension by gently pipetting up and down 5 times. NOTE: This ensures that the cells are evenly dispersed after sitting.
20. Draw up all 500 µL of cell suspension and pipette carefully into the center of the LS column without touching sides of the column.
21. 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 20. 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.
22. 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 "CD34 fraction."

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23. Cap the "Flow Through" tube, this is your CD34 depleted fraction. You may work with this CD34 depleted sample for other downstream processes that this protocol does not cover.
- 24. Add 5 mL of cold RoboSep to the LS column. CRITICAL: Be careful not to touch the sides.
- 25. 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.
26. Set LS Column back on the "CD34 fraction" tube.
- CRITICAL: Do not allow the plunger to interact with external contaminants. It will be used for one more step.
27. Loosen up plunger. Remove plunger briefly from column and hold in one hand.
28. Add another 4 mL of cold RoboSep to the LS Column.
- 29. 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.
30. Discard LS Column and plunger.
31. Centrifuge "CD34 fraction" tube for 10 minutes at 300 rcf.
32. After cells are centrifuged, check for cell pellet.
- 33. Aspirate RoboSep buffer from "CD34 fraction" tube. TIP: Be careful to not aspirate cell pellet.
- 34. Use pipette to aspirate the remaining supernatant from each tube. TIP: Be careful to not aspirate cell pellet.
- 35. Add 1 mL complete RPMI to "CD34 fraction" tube and resuspend cell pellet. TIP: Make sure there are no clumps or bubbles.
- 36. Add an additional 1 mL of complete RPMI and mix thoroughly by gently pipetting up and down 5 times. TIP: Make sure there are no clumps or bubbles.
- 37. Aliquot 10 µL of the CD34 cells into a Lo-Bind Microcentrifuge tube and proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.
38. Move "CD34 fraction" tube to incubator until Cell Stimulation (Chapter 5).

Chapter 5: Cell Stimulation

Materials Required

Complete RPMI (37°C)
R848 (Resiquimod, aliquot at -20°C)
96 Well Flat-Bottom Plate
15 mL Centrifuge Tube with CD34 in complete RPMI

All the following steps should take place in a sterile tissue culture hood.

Methods

1. Retrieve single use aliquot of R848 from -20°C and thaw at room temperature.

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- 2. After R848 is completely thawed, vortex R848 at a slow speed for 10 seconds. TIP: Ensure contents are well suspended.
- 3. Spin R848 in a mini centrifuge for 10 seconds. TIP: Ensure that contents are all in the bottom of the vial.

4. Prepare Complete RPMI Unstimulated media.

    a. Aliquot 5 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.

- CRITICAL: Do not add stimulants into this complete RPMI. Volume required is dependent on number of cells.

- 5. Prepare R848 and complete RPMI mixture by supplementing complete RPMI with 1 µg/mL of R848. CRITICAL: Volume is dependent on number of cells.

    a. Add 1 µL of 1 mg/mL R848 into complete RPMI for every mL of cell suspension needed. This yields a final concentration of R848 of 1 µg/mL.

    b. Use serological pipette to mix thoroughly.

- CRITICAL: Discard remaining/unused R848 — it is single use only and cannot be refrozen.

6. Take CD34 cells from incubator.

7. Centrifuge CD34 cells for 10 minutes at 300 rcf.

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

- 9. Aspirate supernatant with pipette. TIP: Make sure to use a manual pipette to prevent accidental aspiration of cell pellet.
- 10. Using the complete RPMI set aside for the unstimulated condition, resuspend all the CD34 cells with complete RPMI to a cell concentration of 1 x 10^6 cells/mL. TIP: This step is for preparing the unstimulated (negative control) cells. This complete RPMI is not supplemented with any stimulants.
- 11. Plate 100 µL of the CD34 unstimulated cells, per well, on the 96 well flat bottom plate. TIP: Plate at least two wells on the 96 Well Flat Bottom Plate for the unstimulated condition.

12. Centrifuge the remaining CD34 cells (that will serve as the R848 stimulated cells) for 10 minutes at 300 rcf.

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

- 14. Aspirate supernatant with pipette. TIP: Make sure to use a manual pipette to prevent accidental aspiration of cell pellet.

15. Use a pipette to mix the R848 and complete RPMI mixture to ensure it is evenly distributed.

- 16. Using the R848/complete RPMI mixture from step 15, resuspend CD34 cells to a cell concentration of 1 x
- 10^6 cells/mL. CRITICAL: When resuspending, take into consideration the cells removed for the unstimulated condition. TIP: Resuspend as thoroughly as possible, but gently.

- 17. Mix CD34 cells by pipetting up and down gently 5 times. Add 100 µL of cell suspension per empty well on 96 well flat-bottom plate. CRITICAL: Be careful not to create bubbles. This will maximize even stimulation of cell suspension.

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18. 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 thaw, prepare liquid reagents and setup in the Bruker instrument. Refer to your instrument's system guide for detailed instructions.

Chapter 7: Cell Staining

Materials Required

Stimulated and Unstimulated CD34 Cells in 96 Well Plate
3 x Lo-Bind Microcentrifuge Tube (*Stain Master Mix, CD34, Cell Count*)
Sterile 1X PBS (Room Temperature)
Complete RPMI (37°C)
Cell Stain 405 (-20°C)
Cell Stain 405 Diluent (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. Prepare cell stain 405 stock.
    a. Thaw tube of cell stain 405 diluent (DMSO) at room temperature.
    b. Spin tubes of cell stain 405 and cell stain 405 diluent (DMSO) in a mini centrifuge for 10 seconds to collect the contents at the bottom of the tubes.
    c. Add 20 µL of cell stain 405 diluent (DMSO) directly to the tube of cell stain 405. Pipet up and down 15 times gently to resuspend.

- CRITICAL: Cell stain 405 must be prepared fresh. Discard remaining stain – do not store.

- 2. Prepare stain master mix by diluting 2 µL of cell stain 405 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 cell stain 405 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.

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a. With a P1000 set to 500 µL, 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.**

3. Remove 96 well plate with CD34 cells from incubator.
4. Mix CD34 cells by pipetting up and down. Transfer cells to a Lo-Bind microcentrifuge tube by using P100 pipette to draw up 100 µL at a time in a gentle, circular motion until well is empty. **NOTE: Pool wells if there are replicates.**
5. Centrifuge cells for 10 minutes at 300 rcf.
6. After cells are centrifuged, check for cell pellets.
- 7. 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 pellets.**

   ***NOTE: Supernatants may be stored at -80°C for bulk assay.**

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

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

9. Centrifuge cells for 10 minutes at 300 rcf.
10. After cells are centrifuged, check for cell pellets.
- 11. 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 pellets.**
12. Gently remix stain master mix.

- **CRITICAL: Failure to remix stain master mix will result in poor staining.**
- 13. For every 1 x 10^6 cells, add 100 µL of **well mixed** stain master mix to each cell suspension tube. **CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.**
14. Incubate for 5 minutes at 37°C in the dark.
- 15. Gently pipet to mix the cell suspension **15 times**. **CRITICAL: Be careful to not create bubbles.**
16. Incubate for an additional 5 minutes at 37°C in the dark.
- 17. After incubation, add 5 times the volume of complete RPMI. **CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.**
18. Incubate for 10 minutes at 37°C in the dark.
- 19. 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.**
20. Centrifuge stained cells for 10 minutes at 300 rcf.
21. After cells are centrifuged, check for cell pellets.
- 22. Aspirate supernatant with a pipette. **TIP: Be careful not to aspirate the cell pellets.**

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23. Resuspend the cell pellet with complete RPMI to a cell density of 7.5 x 10^5 cells/mL. Proceed to Chapter 8.

Chapter 8: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag
Stained CD34 Cells at 7.5 x 10^5 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 unstimulated and stimulated CD34 cells by gently pipetting up and down. 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.
3. Let IsoCode chips sit for one minute on a flat surface.
4. Check bottom of chip to ensure liquid has entered the chip. TIP: If liquid has not flowed, tap IsoCode chip on flat surface lightly.
5. When inserting IsoCode chip into the 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

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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 1X 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]

D2 Protocol: Dead Cell Removal Using Ficoll

Materials Required

Complete RPMI (37°C) Cells (Minimum 3x10^6)

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2 x 15 mL Centrifuge Tubes
Lo-Bind Microcentrifuge Tube(s)
Ficoll Paque

- CRITICAL: It is recommended to start this protocol with a minimum of 3 x 10^6 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.
    a. 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 10^7 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.

D3 Protocol: Dead Cell Removal Using Low Speed Spin

Materials Required

Complete RPMI (37°C)
Cells (Maximum 3x10^6)

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15 mL Centrifuge Tube
Lo-Bind Microcentrifuge Tube(s)

- CRITICAL: It is recommended to use this protocol over Ficoll (Appendix D2) only when both viability and cell counts are low.

1. Centrifuge cells at 300 rcf for 10 minutes. Aspirate supernatant and resuspend the pellet in 3-6 mL of complete RPMI. This can be done in the same 15 mL centrifuge tube used to initially harvest cell culture(s).
2. Centrifuge 15 mL centrifuge tube at 150 rcf for 5 minutes at room temperature.

- CRITICAL: These steps must be done carefully and slowly to avoid excessive healthy cell loss.

3. Allow the centrifuged tube to rest, undisturbed, for 5 minutes at room temperature.
4. Remove the dead cells by carefully and slowly aspirating the supernatant, without disturbing the cell pellet.
5. Resuspend cells in 500 µL of complete RPMI.

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

Troubleshooting & References

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Problem	Possible Reason	Solution
Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Viability related*	• Leaving thawed cells in DMSO for an extended period • Low viable cells due to low viability input sample and lack of utilization of Ficoll Paque or low speed spin • Decreased viability due to cell shock	• After thaw, quickly transfer cells from DMSO to complete RPMI to ensure viability of cells. • Verify viability of cells is above 80% as stated in **Chapter 3** to ensure protocol is being performed with the highest quality of cells. Use Ficoll Paque in **Appendix D2** or low speed spin in **Appendix D3** If viability is less than 80%
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	• Use recommended cell concentrations during loading (**Chapter 5**) • Use recommended temperatures (i.e. always use warmed media [37°C]) • Use appropriate dilutions recommended in **Appendix D1** • Use cell concentration listed in **Chapter 5** • Use recommended vendor as listed in **Table 3**
Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Stimulation step related*	• Recommended R848 stimulation concentration was not used • Recommended R848 stimulation duration was not used • Poor cell retrieval from plate	• Use R848 at final concentration listed in **Chapter 5** • Use recommended R848 stimulation duration listed in **Chapter 5** • 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 the cells • Thoroughly mix cells in well with pipette prior to transferring to tube (refer to **step 7.4**)
Low quality cell count on chip *Stain Process related*	• Use of media other than the recommended media in protocol which could interact with cell stain • Use of stains not recommended in protocol • Recommended stain concentration, incubation time and/or incubation temperature not used • Cell stain 405 was stored prior to use • Media not completely removed from cell pellet prior to staining	• Use complete RPMI media following recipe in **Table 7** • Use Bruker provided validated stain (**Table 4: Cell Staining Reagents**) • Follow staining steps as highlighted in **Chapter 7** • Use only freshly prepared cell stain 405 per **Chapter 7** • Ensure all media is removed from cell pellet in **step 7.7**

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 step in **8.2** 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 4** (CD34 Sample Enrichment)
		• Load recommended number of cells (30,000 cells per chip) (**Chapter 8**)
		• Use low protein binding centrifuge tubes

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

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