

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

CANCER RESEARCH ★ DRUG DISCOVERY OMICS

IsoPlexis Applications Team, IsoPlexis

Aug 09, 2021

Abstract

A. Overview

1. 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.

Day 3: 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 IsoPlexis will require validation prior to use. Please consider IsoPlexis' IsoPACE™ program to assist in custom marker and protocol validation.

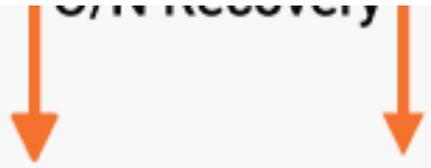
1. Sample Enrichment

Thaw cells with IL-2

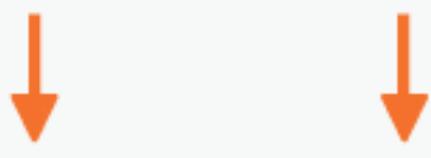
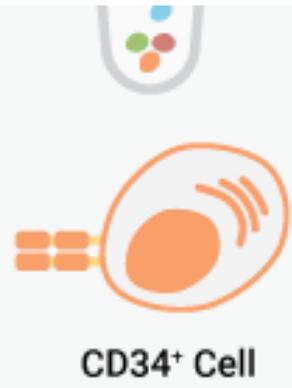
O/N Recovery



CD34⁺ recovery

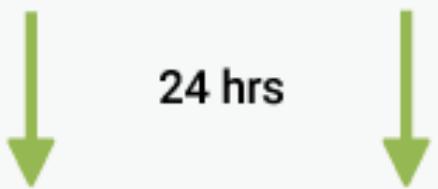
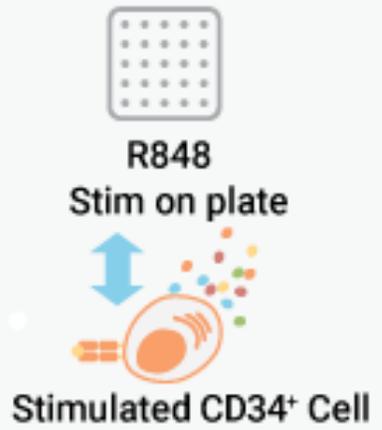


Enrich for CD34⁺ Cells



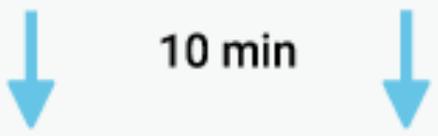
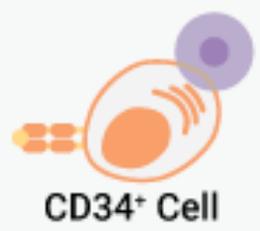
2. Cell Stimulation

Stim on Plate with R848



3. Cell Staining

Add Cell Stain 405 to CD34⁺ Cells



4. Load Cells

Load onto IsoLight



Introduction

2. 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

3. Required Reagents, Consumables and Equipment

Table 1: Required Reagents and 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
 - Cell Stain 405 [ordered separately]
 - Cell Stain 405 Diluent (DMSO) [part of cell stain 405 kit]

Table 2: Required Consumables Not Supplied by IsoPlexis

Consumable	Type	Source	Catalog Number
T25 Flask	N/A	Corning	353108
T75 Flask	N/A	Corning	430641U
6 Well Plate Flat-Bottom	N/A	Corning	3516
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

Table 3: Reagents Required to Perform Protocol but 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
Recombinant IL-2	200 µg/mL	Biologend	589104
Bovine Serum Albumin (BSA), lyophilized powder	N/A	Sigma-Aldrich	A9647-10G
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
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	tlr-r848
Endotoxin-free Water	1x	InvivoGen	tlr-r848
Peripheral Blood Mononuclear Cells (PBMCs) or Bone Marrow Mononuclear Cells (BMNCs)		Experiment Specific	
Reagent alcohol 70%		VWR	BDH1164-4LP

Table 4: Cell Staining Reagents

Test Material	Catalog Number	Color
Cell Stain 405	STAIN-1001-1	Violet

Table 5: Required Equipment

Equipment	Source	Catalog Number
IsoLight, IsoSpark, or IsoSpark Duo Instrument	IsoPlexis	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.

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 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 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 Solution Stabilized	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 0.20 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
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*	Invitrogen/20012-027

*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 25 μL R848 aliquots and freeze at -20°C for no longer than 6 months. Avoid repeated freeze thaw cycles.

C. Protocol

Chapter 1: Getting Started

Kit Contents:

- 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
 - 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 $\mu\text{g}/\text{mL}$ (-20°C)
- Cryopreserved PBMC or BMNC
- 15 mL Centrifuge Tube
- Plate and/or Flask
 - For $> 10^6$ M cells, T75 Flask
 - For $6 - 9.9 \times 10^5$ M cells, T25 Flask
 - For $< 6 \times 10^5$ 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.**
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 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. 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.

11. After cells are centrifuged, check for cell pellet.
12. Aspirate supernatant. **TIP: Be careful not to aspirate cell pellet.**
 - Use pipette to remove last bit of supernatant.
13. 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.**
14. Slowly add additional complete RPMI to a final concentration of 1×10^6 cells/mL.
15. Mix thawed IL-2 thoroughly by carefully pipetting up and down.
16. 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.

17. Mix with serological pipette. **TIP: Gently pipet up and down 3-5 times, be careful to not create bubbles.**
18. Transfer cell suspension to flask or plate. **TIP: Slowly pipette down the side of the flask as to not create bubbles.**
19. 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.**
20. Move to incubator for overnight recovery at 37°C, 5% CO₂.

Chapter 3: Pre-Sample Enrichment

Materials Required:

- 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 μ L 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.**

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.**
7. Proceed immediately to next chapter.

Chapter 4: CD34 Sample Enrichment

Materials Required:

- Complete RPMI (37°C)
- 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.**
 - Use pipette to aspirate remaining supernatant.

CRITICAL: For every 1 x 10⁸ 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 10⁸ 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 10⁸ 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.
 9. After 30 minutes, add 7 mL of cold RoboSep for 1 x 10⁸ 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.

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 10⁸ or fewer cells, resuspend with 500 µL of cold RoboSep.

- 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 10⁸ 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 μ L to ensure all 500 μ L 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 μ L 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.

- 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.**
- 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.**

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."

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 aliquot of R848 from -20°C and thaw at room temperature. **CRITICAL: Aliquots are suggested as single use.**

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 R848 and complete RPMI mixture by supplementing complete RPMI with 1 $\mu\text{g}/\text{mL}$ of R848. **CRITICAL: Volume is dependent on number of cells.**

- 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 $\mu\text{g}/\text{mL}$.
- Use serological pipette to mix thoroughly.

5. Take CD34 cells from incubator.

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

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

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

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

10. Using the R848/complete RPMI mixture from step 9, resuspend CD34 cells to a cell concentration of 1×10^6 cells/mL. **TIP: Resuspend as thoroughly as possible, but gently.**

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

12. 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 IsoPlexis instrument. Refer to your instrument's system guide for detailed instructions.

Chapter 7: Cell Staining

Materials Required:

- Stimulated 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.
 - Thaw tube of cell stain 405 diluent (DMSO) at room temperature.
 - 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.
 - 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.**

- 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.**

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. Aspirate supernatant with a pipette.* **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.
9. Centrifuge cells for 10 minutes at 300 rcf.
10. After cells are centrifuged, check for cell pellets.
11. Aspirate supernatant with a pipette.* **TIP: Be careful not to aspirate the cell pellets.**
12. For every 1×10^6 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. Gently remix master mix if it has been sitting for longer than a few minutes.**
13. Incubate for 5 minutes at 37°C in the dark.
14. Gently pipet to mix the cell suspension 15 times. **CRITICAL: Be careful to not create bubbles.**
15. Incubate for an additional 5 minutes at 37°C in the dark.
16. After incubation, add 5 times the volume of complete RPMI. **CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.**
17. Incubate for 10 minutes at 37°C in the dark.
18. 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.**
19. Centrifuge stained cells for 10 minutes at 300 rcf.
20. After cells are centrifuged, check for cell pellets.
21. Aspirate supernatant with a pipette. **TIP: Be careful not to aspirate the cell pellets.**
22. Resuspend the cell pellet with complete RPMI to a cell density of 7.5×10^5 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 7.5×10^5 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 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 IsoPlexis 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.

Notes and Comments

D: Appendix

D1 Protocol: Cell Quantification & Viability

Materials Required:

- Hemocytometer
- 10 μ L 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 μ L 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 1X 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) from D.1.4 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]

D2 Protocol: Dead Cell Removal Using Ficoll

Materials Required:

- Complete RPMI (37°C)
- Cells (Minimum 3x10⁶)
- 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 10⁶ 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 1×10^7 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 μ L 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 3×10^6)
- 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 μ L of complete RPMI.

6. Aliquot 10 μ L 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.**

For troubleshooting, see page 23 on the [PDF of the protocol](#), or contact Support at 475-221-8402 & support@isoplexis.com with specific troubleshooting questions.

References

IsoPlexis