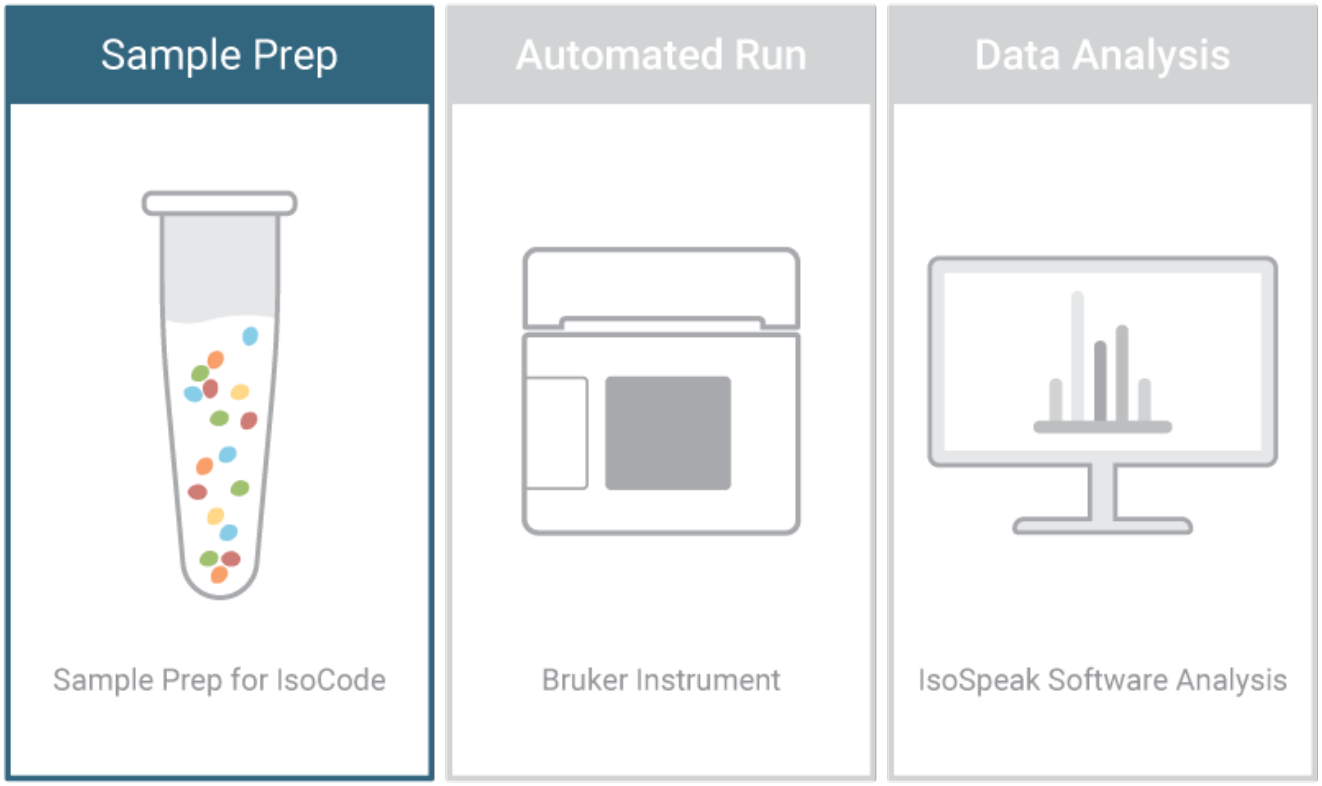
IsoCode Single-Cell Adaptive Immune: Human PBMC Protocol with CytoStim

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



Key: ● TIP, ● CRITICAL, ● OPTIONAL

Contents

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A. Overview

Overview of Protocol

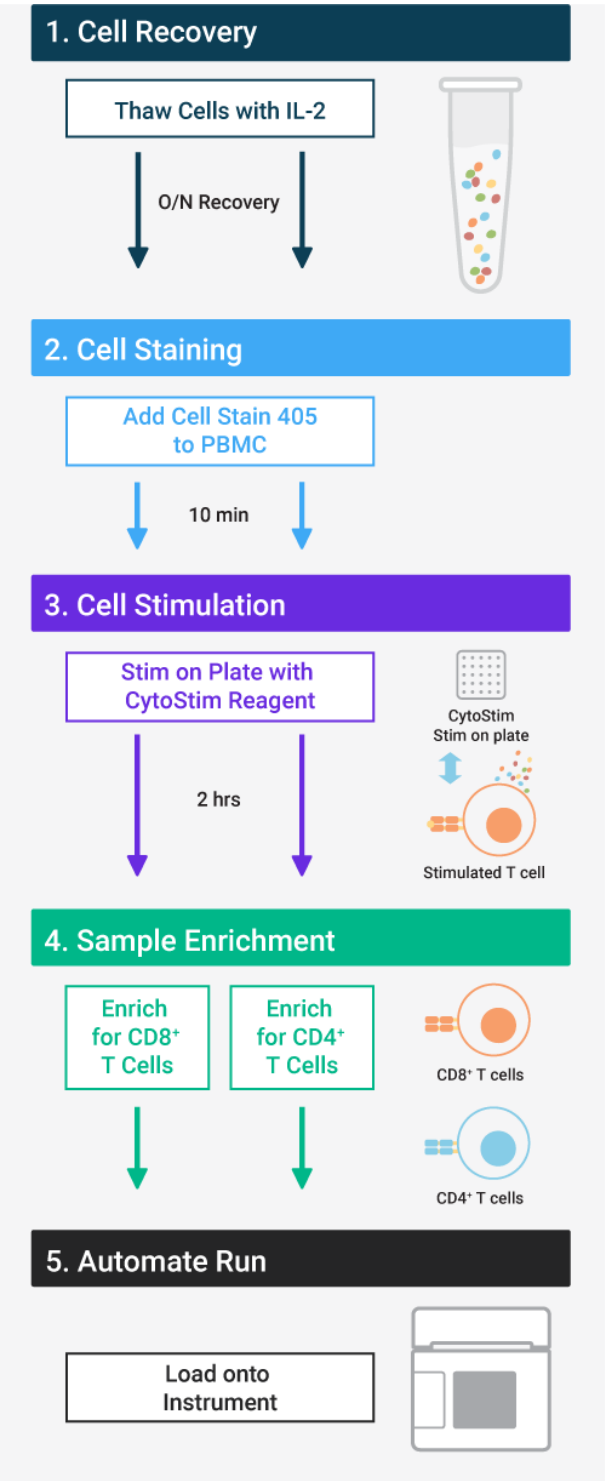
Day 1: Cryopreserved cells are thawed and cultured overnight in the presence of IL-2.

Day 2: Staining and Stimulation of cells for 2 hours. Enrichment and Loading of T cells onto IsoCode chip.

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

NOTE:
Using stains and protocols other than recommended stain 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.

NOTE:
This protocol requires starting with human PBMCs and the cell population cannot be purified prior to stimulation. CytoStim Reagent requires antigen presenting cells (APCs) to be present for the stimulation to occur.



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PRO-47 REV 4.0

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 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
6 Well Plate Flat Bottom	N/A	Corning	353046
12 Well Plate Flat Bottom	N/A	Corning	353043
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	62406-200
Centrifuge Tubes*	50 mL	VWR	21008-242
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

*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
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
CytoStim, Human	50x	Miltenyi	130-092-172
RoboSep buffer	1x	StemCell Tech	20104
Miltenyi CD8 Microbeads, Human, 2mL	N/A	Miltenyi	130-045-201
Miltenyi CD4 Microbeads, Human, 2mL	N/A	Miltenyi	130-045-101
Trypan Blue	0.4%	Gibco	15250-061
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
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	02-671-51B
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

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 comes 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
Bovine Serum Albumin (BSA), lyophilized powder	N/A	1%	1 g	Sigma-Aldrich/ A9647-10G
Phosphate Buffered Saline (1X PBS) 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 8
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.

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
- 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
- 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. Centrifugation steps should be performed at 21°C.

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 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 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 2 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

Sterile 1X PBS (Room Temperature)
50 mL Centrifuge Tube
Overnight Recovered Cells from Chapter 2 or
Fresh PBMC if Working with Fresh Samples
Lo-Bind Microcentrifuge Tube for Cell Count

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

Methods

1. Transfer cells from flask or plate into 50 mL centrifuge tube.
- 2. Add 1X PBS to flask or plate and rinse 5 times. TIP: Make sure to spread out the 1X PBS to gather maximum number of cells.
    - For T75 Flask add 3 mL
    - For T25 Flask add 2 mL
    - For 6 Well Plate add 1 mL
3. Transfer cell/1X PBS mixture to the 50 mL centrifuge tube.
4. Centrifuge cells for 10 minutes at 300 rcf.
5. Remove the centrifuged cells and check for cell pellet.
- 6. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    - Use pipette to aspirate remaining supernatant.
7. Add 5 mL 1X PBS to 50 mL centrifuge tube and resuspend cell pellet.
- CRITICAL: Failure to remove excess media will result in poor staining in Chapter 4.
- 8. 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.
- 9. 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.
10. Proceed immediately to next chapter.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Chapter 4: Cell Staining

Materials Required

Prepared Cells from Chapter 3 3 x 15 mL Centrifuge Tubes (*Stain Master Mix, Unstim PBMC, CytoStim PBMC*) 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 16 µL of cell stain 405 into 8 mL of 1X PBS in a 15 mL centrifuge 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.
    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.**

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

● 4. 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.

5. Gently remix stain master mix.

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

● 6. For every 1 x 10^7 cells, add 1 mL of **well mixed** stain master mix to the 50 mL centrifuge tube. CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.

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

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

9. Incubate for an additional 5 minutes at 37°C in the dark.
10. After incubation, add the same volume of complete RPMI. CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.
11. Incubate for 10 minutes at 37°C in the dark.
12. After incubation, split the cell suspension in half into two new 15 mL centrifuge tubes.
    a. Pipette one half of the cell suspension into a 15 mL centrifuge tube labeled "Unstim PBMC".
    b. Pipette the other half of the cell suspension into a 15 mL centrifuge tube labeled "CytoStim PBMC".

    NOTE: Use the cell count from Chapter 3 step 9 and divide by 2 to get the number of cells in each 15 mL centrifuge tube.
13. Centrifuge stained cells for 10 minutes at 300 rcf.
14. Proceed immediately to next chapter.

Chapter 5: Cell Stimulation

Materials Required

Complete RPMI (37°C)
CytoStim, Human (4°C)
Unstim PBMCs Tube
CytoStim PBMCs Tube
12 Well Plate Flat Bottom
2 x 15 mL Centrifuge Tubes

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

Methods

1. 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) conditions.

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

2. Prepare CytoStim complete RPMI mixture. Aliquot 4.9 mL complete RPMI into a 15 mL centrifuge tube labeled "Cytostim Complete RPMI". CRITICAL: Volume required is dependent on number of cells.
    a. Aliquot 4.9 mL complete RPMI into a 15 mL centrifuge tube labeled "CytoStim Complete RPMI".
    b. Add 100 µL of CytoStim to the complete RPMI.
    c. Use serological pipette to mix thoroughly.
3. After cells are centrifuged, check for cell pellets.

- 4. Aspirate supernatant with pipette. TIP: Make sure to use a manual pipette to prevent accidental aspiration of cell pellet.
- 5. Using the "Complete RPMI Unstimulated" media set aside for the unstimulated condition, resuspend cells in the 15 mL centrifuge tube labeled "Unstim PBMC" to a cell concentration of 5 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.
6. Vortex "CytoStim Complete RPMI" mixture for 5 seconds.
7. Use a pipette to mix the "CytoStim Complete RPMI" mixture to ensure it is evenly distributed.
- 8. Using the "CytoStim Complete RPMI" mixture from step 7, resuspend cells in the 15 mL centrifuge tube labeled "CytoStim PBMC" to a cell concentration of 5 x 10^6 cells/mL. TIP: Resuspend as thoroughly as possible, but gently.
9. Seed each cell suspension into wells on a 12 well plate flat bottom in a manner following the parameters of the table (See Table 10). NOTE: Multiple wells may be plated per condition depending on the volume of the suspension. Label one half of the wells with unstimulated PBMCs as "Unstim CD8" and the other half as "Unstim CD4". Label one half of the wells with stimulated PBMCs as "CytoStim CD8" and the other half as "CytoStim CD4". Labeling the wells with either CD4 or CD8 is in preparation for enriching each fraction in Chapter 7.

Table 10: Plate and Volume for PBMC Seeding

Plate	Minimum Vol. per Well (mL)	Standard Vol. per Well (mL)	Max Vol. per Well (mL)
12 Well Plate	0.8	1	1.5

10. Incubate plate for 2 hours at 37°C, 5% CO_2.

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.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Chapter 7: CD8 & CD4 Sample Enrichment

Materials Required

Complete RPMI (37°C)
RoboSep Buffer (4°C)
Miltenyi CD8 Microbeads, Human, 2 mL (4°C)
Miltenyi CD4 Microbeads, Human, 2 mL (4°C)
4 x MACS LS Column
CytoStim Stimulated & Unstimulated PBMCs in 12 Well Plate from Chapter 5
4 x 15 mL Centrifuge Tubes (Unstim *CD8, Unstim CD4, CytoStim CD8, CytoStim CD4)*
Enrichment Kit:
- 2 x MACS Metal Plate/Magnet Kit
- 12 x 15 mL Centrifuge Tubes (4 x *Discard, 4 x Flow Through, 1 x Unstim CD8, 1 x CytoStim CD8, 1 x Unstim CD4, 1 x CytoStim CD4*)
- 4 x Lo-Bind Microcentrifuge Tubes for Post-Enrichment

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

Methods

1. Remove 12 well plate with PBMCs from incubator.
2. Mix cells by pipetting up and down. Transfer cells to a 15 mL centrifuge tube. NOTE: Pool wells if there are replicates.
    a. From the well(s) labeled "Unstim CD8", transfer cells to a new 15 mL centrifuge tube labeled "Unstim CD8".
    b. From the well(s) labeled "CytoStim CD8", transfer cells to a new 15 mL centrifuge tube labeled "CytoStim CD8".
    c. From the well(s) labeled "Unstim CD4", transfer cells to a new 15 mL centrifuge tube labeled "Unstim CD4".
    d. From the well(s) labeled "CytoStim CD4", transfer cells to a new 15 mL centrifuge tube labeled "CytoStim CD4".
3. Centrifuge cells for 10 minutes at 300 rcf.
4. Remove the centrifuged cells and check for cell pellets.
5. Aspirate supernatant*. TIP: Since it is a small volume, use pipette for this step to prevent accidental aspiration of the cell pellet.

    *NOTE: Supernatants may be stored at -80°C for bulk assay. Supernatant will contain secretions from T cells as well as other cells within PBMCs.

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

6. Add 80 µL of cold RoboSep to each 15 mL centrifuge tube containing 1 x 10^7 or fewer cells.

7. Vortex the Miltenyi CD8 and CD4 Microbeads at a slow speed for 10 seconds.
8. Add 20 µL of the appropriate Miltenyi Microbeads and mix well by gently pipetting up and down 5 times.
    a. Add 20 µL of Miltenyi CD8 Microbeads to the 15 mL centrifuge tube labeled "Unstim CD8".
    b. Add 20 µL of Miltenyi CD8 Microbeads to the 15 mL centrifuge tube labeled "CytoStim CD8".
    c. Add 20 µL of Miltenyi CD4 Microbeads to the 15 mL centrifuge tube labeled "Unstim CD4".
    d. Add 20 µL of Miltenyi CD4 Microbeads to the 15 mL centrifuge tube labeled "CytoStim CD4".

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

- 9. Incubate in refrigerator (4°C) for 15 minutes. TIP: Don't incubate on ice as increased incubation times may be required.
10. After 15 minutes, add 2 mL of cold RoboSep to each 15 mL centrifuge tube.
- 11. Centrifuge cells for 10 minutes at 300 rcf.
- TIP: Keep RoboSep in refrigerator during enrichment process.

NOTE: Steps below describe a parallel workflow for unstimulated PBMCs that will be enriched for CD8 cells, stimulated PBMCs that will be enriched for CD8 cells, unstimulated PBMCs that will be enriched for CD4 cells, and stimulated PBMCs that will be enriched for CD4 cells.

- 12. Set up MACS sorting by setting metal plates in tissue culture hood and placing magnets on each 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. NOTE: Label each of the 4 LS columns for the appropriate condition.
13. After cells are centrifuged, check for cell pellet and continue with MACS separation.
- 14. Aspirate supernatant from cell pellet. TIP: Since it is a small volume, use pipette for this step to prevent accidental aspiration of the cell pellet.
15. For 1 X 10^8 or fewer cells, resuspend each cell pellet 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.
- 16. Start with each LS column over a "Discard" tube, add 3 mL of cold RoboSep to each 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 next step. As a reminder, be careful to not let the column dry out.
17. Unscrew and keep cap for each "Flow Through" tube. NOTE: This is in preparation for next step to ensure the column does not dry out during the transition.
- 18. When last drop falls through to "Discard" tube, move the rack over so each LS column is over a "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.

19. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.
20. Mix each cell suspension by gently pipetting up and down 5 times. NOTE: This ensures that the cells are evenly dispersed after sitting.
21. Draw up all 500 µL of cell suspension and pipette carefully into the center of the LS column without touching sides of the column. Repeat this step for each cell suspension.
22. Wash each LS column 3 times with 3 mL of cold RoboSep.
    a. First wash: Rinse inside walls of cell suspension tube with 3 mL of RoboSep before transferring the mixture to LS Column. NOTE: This is to retrieve any cells that have been left behind.
    i. Pipette all of the mixture into LS Column after last drop passes through or does not fall from step 21. 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.
23. 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 appropriately for each condition. NOTE: At this point, one column, with the corresponding cells, will be placed on a new tube for each of the following conditions: unstimulated cells labeled with CD8 Microbeads on a 15 mL centrifuge tube labeled "Unstim CD8", stimulated cells labeled with CD8 Microbeads on a 15 mL centrifuge tube labeled "CytoStim CD8", unstimulated cells labeled with CD4 Microbeads on a 15 mL centrifuge tube labeled "Unstim CD4", and stimulated cells labeled with CD4 Microbeads on a 15 mL centrifuge tube labeled "CytoStim CD4".
24. Cap each "Flow Through" tube and discard.
25. Add 5 mL of cold RoboSep to the LS Column. CRITICAL: Be careful not to touch the sides.
26. 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.
27. Set LS Column back on the appropriate 15 mL tube.
28. Repeat steps 25 to 27 for each LS Column.
29. Loosen up plunger. Remove plunger briefly from column and hold in one hand.

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

30. Add another 4 mL of cold RoboSep to the LS Column.
31. 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.
32. Discard LS Column and plunger.
33. Repeat steps 29 to 32 for each LS Column.

NOTE: There are now four 15 mL centrifuge containing cells from each condition: unstimulated CD8 fraction, stimulated CD8 fraction, unstimulated CD4 fraction, and stimulated CD4 fraction.

- 34. Mix well 5 times with 10 mL serological pipette. TIP: Be careful not to create bubbles.
- 35. Aliquot 10 µL of each fraction into a Lo-Bind microcentrifuge tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
- 36. 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.
- 37. After cells are centrifuged, check for cell pellets.
- 38. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
- 39. Resuspend each cell pellet with complete RPMI to a cell density of 1 x 10^6 cells/mL. Proceed to Chapter 8.

Chapter 8: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 6
Unstimulated CD8 Cells at 1 x 10^6 cells/mL
Stimulated CD8 Cells at 1 x 10^6 cells/mL
Unstimulated CD4 Cells at 1 x 10^6 cells/mL
Stimulated CD4 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 CD8 fractions by gently pipetting up and down 15 times. Immediately proceed to chip loading. Pipette 30 µL of each cell suspension into individual IsoCode chips. 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.
- 3. Resuspend CD4 fractions by gently pipetting up and down 15 times. Immediately proceed to chip loading. Pipette 30 µL of each cell suspension into individual IsoCode chips. 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 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 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 may be used in place of manual cell counting. Please pay close attention to any stains that have already been applied to cells as they may not be spectrally compatible with automated cell counting stains. If you are unsure, it is recommended to use manual cell counting.

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]

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 5**) • 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 the cells • Thoroughly mix cells in well with pipette prior to transferring to tube (refer to **step 7.2**)
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 4** • Use only freshly prepared cell stain 405 per **Chapter 4** • Ensure all media is removed from cell pellet in **step 4.4**

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** and **8.3** 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 8**
		• Load recommended number of cells (30,000 cells per chip) (**Chapter 8**)
		• Use low protein binding centrifuge tubes

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])

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Stimulation step related*	• Recommended CytoStim stimulation concentration was not used • Recommended CytoStim stimulation duration was not used	• Use CytoStim concentrations listed in **Chapter 5** • Use CytoStim timing listed in **Chapter 5** • Use recommended vendor as listed in **Table 3**