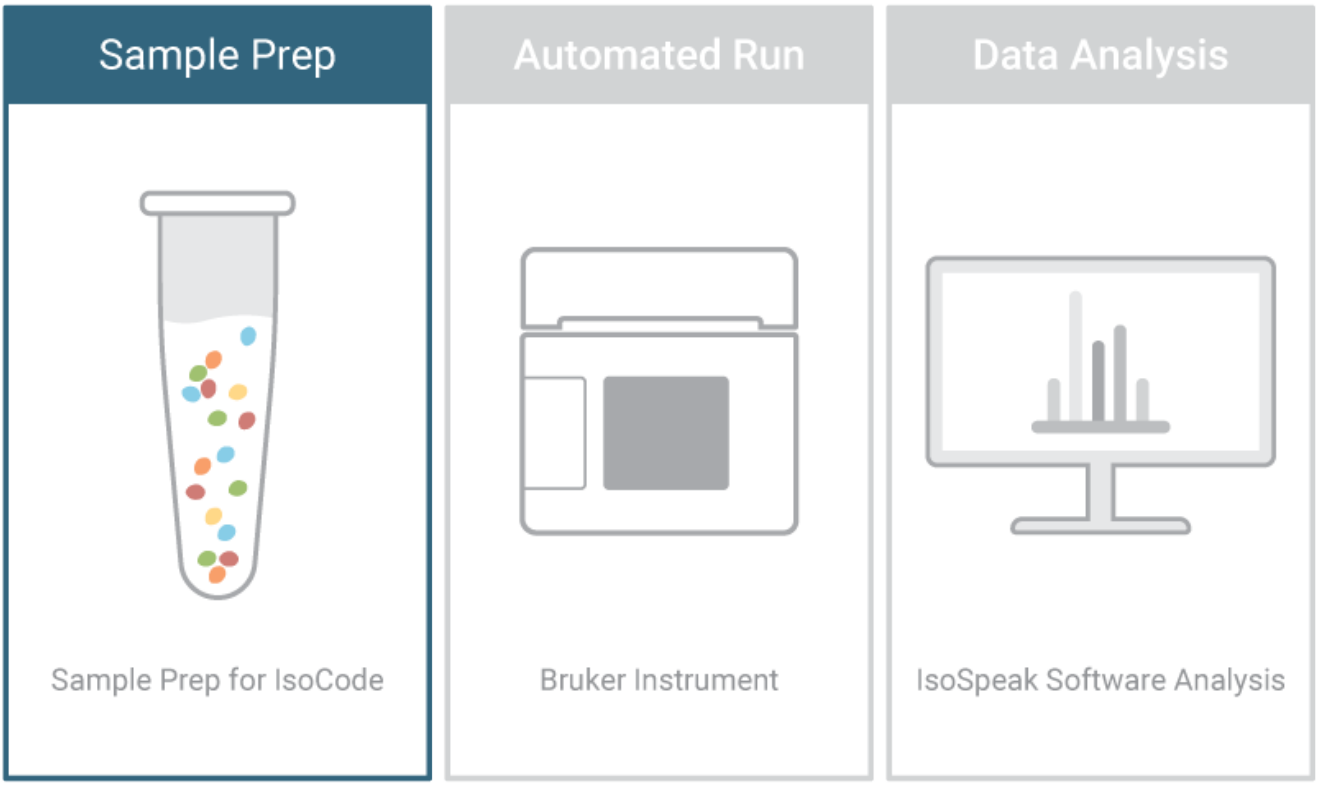
IsoCode Single-Cell Adaptive Immune: Human Bispecific Protocol

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.

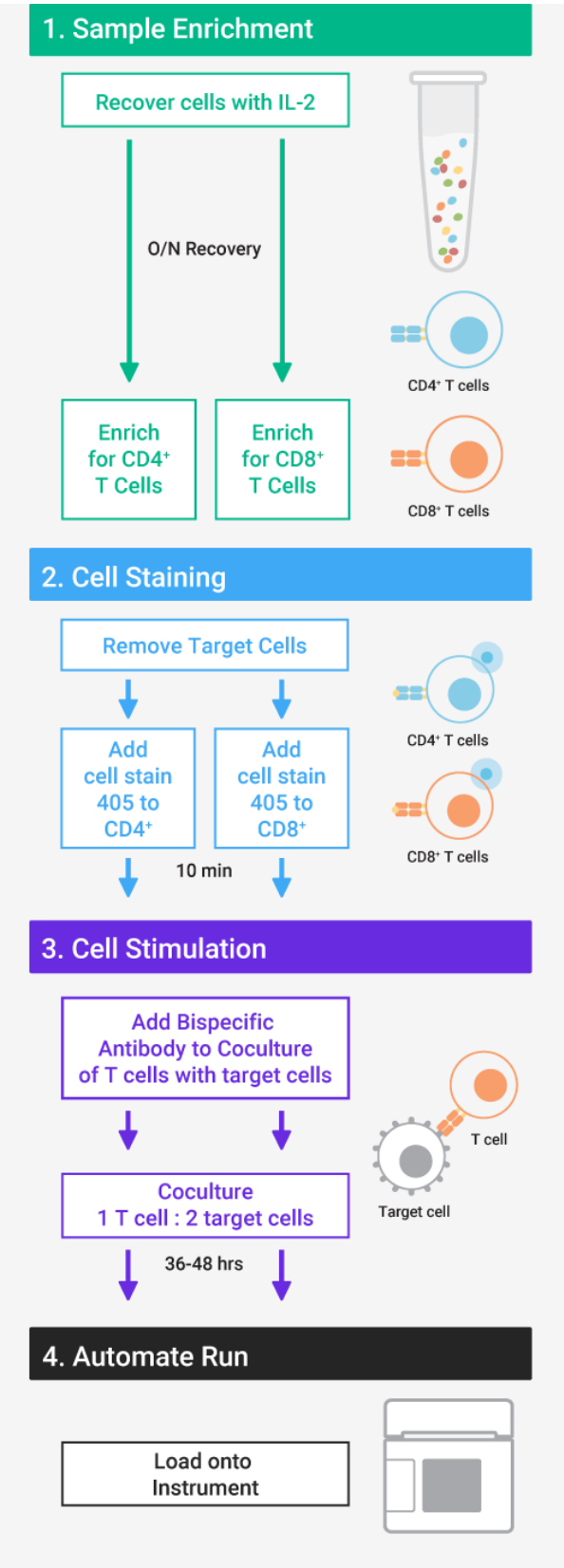
Day 2: Enrichment, Staining and Antigen Stimulation of CD4+ and/or CD8+ T cells for 36-48 hours.

Day3: 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:
A bispecific antibody concentration applicable to your experimental design should be selected prior to starting this protocol. Please contact your Field Application Scientist for assistance with experimental design.

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

- Read MSDS documents of all materials prior to use.

- Laboratory workers should wear standard PPE, including disposable gloves, protective eyewear, and laboratory coats.

Required Reagents, Consumables and Equipment

Table 1: Required Consumables Provided by Bruker

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

IsoCode Kit Components

IsoLight IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 1.5 mL Tubes A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
- 50 mL Tubes containing Reagents 1, 2, 3, 4, 5, 6, 7, 8
- 1 Bag of Disposable Reagent Sippers

IsoSpark IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 1.5 mL Tubes A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
- Cartridge containing Reagents 1, 2, 3, and 4

IsoCode Chip Set (-20°C)

- Boxes of IsoCode Chips (2 per box)
  - IsoSpark: 4 chip kits
  - IsoLight: 4 or 8 chip kits
- 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
T25 Flask	N/A	Corning	353108
T75 Flask	N/A	Corning	430641U
6 Well Plate Flat Bottom	N/A	Corning	353046
96 Well Plate U-Bottom	N/A	Corning	353077
MACS LS Column	N/A	Miltenyi	130-042-401
Centrifuge Tubes*	Polypropylene, 15 mL	VWR	62406-200
Pipette Tips (Filtered)	10 µL Graduated Filter Tips 100 µL Graduated Filter Tips 1000 µL XL Graduated Filter Tips	USA Scientific	1181-3710 1183-1740 1182-1730
Serological Pipette	2 mL Pipette 5 mL Pipette 10 mL Pipette	USA Scientific	1072-0510 1075-0110 1071-0810
Lo-Bind Microcentrifuge Tubes, Sterile	1.5 mL	USA Scientific	4043-1081
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
Polystyrene Round Bottom Tube	5 mL	Falcon	352058

*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
Robosep buffer	1x	StemCell Tech	20104
Ficoll Paque Plus	N/A	GE Healthcare	17-1440-03
Miltenyi CD8 Microbeads, Human, 2 mL	N/A	Miltenyi	130-045-201
Miltenyi CD4 Microbeads, Human, 2 mL	N/A	Miltenyi	130-045-101
Trypan Blue	0.4%	Gibco	15250-061
Bi-Specific antibody		Experiment dependent	
Control antibody		Experiment dependent	
Target cells		Target-type dependent	
Reagent alcohol 70%	N/A	Lab Grade	N/A
Dynabeads M-280 Streptavidin	N/A	Thermo-Fisher	11205D (2 mL)
Biotin anti-human CD235a clone: HIR2 (GA-R2) – K562 Cell Line	0.5 mg/ml	Invitrogen	13-9987-82
Biotin eBioscience anti-human CD19 Monoclonal Antibody clone: HIB19 – Raji Cell Line	0.5 mg/ml	Invitrogen/Thermo-Fisher-Scientific	13-0199-82

*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/Requirements
IsoLight, IsoSpark, or IsoSpark Duo Instrument	Bruker	ISOLIGHT-1000-1, ISOSPARK-1000-1, or ISOSPARK-1001-1
Culture Hemocytometer	(Fisher) Hauser Levy	02-671-55A
Hemocytometer cover glass	(Fisher) Hauser Levy	02-671-53
MidiMACS Separator	Miltenyi	130-042-302
MACS MultiStand	Miltenyi	130-042-303
EasySep magnet	StemCell Technologies	18000

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 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
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: 0.1% BSA Recipe

Ingredient	Stock Concentration	Final Concentration	Amount for 100 mL	Vendor/Catalog
Bovine Serum Albumin (BSA), lyophilized powder	N/A	0.1%	0.1 g	Sigma-Aldrich/ A9647-10G
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1X	1X	99.9 mL initially*	Gibco/10010072

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

Table 10: 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.

Bulk Depletion Bead Preparation

- CRITICAL: Prepare target cell line depletion beads prior to starting this protocol. See Appendix D3 for instructions. Appendix D3 describes the protocol for how to conjugate depletion beads for two widely used target cells; K562 cells (CD235a) and Raji cells (CD19). These depletion beads can be used for any target cells expressing CD235a or CD19 respectively.

C. Protocol

Chapter 1: Getting Started

Kit Contents

IsoLight IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 15 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 Kit]

Chapter 2: Recovery of Cryopreserved Target Cell Lines

Materials Required

Complete RPMI (37°C)
15 mL Centrifuge Tube (*Target Cells*)
Cryopreserved Target Cell Lines
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

- CRITICAL: Prepare target cell line depletion beads prior to starting this protocol. See Appendix D3 for instructions. Appendix D3 describes the protocol for how to conjugate depletion beads for two widely used

target cells; K562 cells (CD235a) and Raji cells (CD19). These depletion beads can be used for any target cells expressing CD235a or CD19 respectively.

- CRITICAL: This step should be performed at least three days prior to the recovery of the T cells (Chapter 4).

1. Pipette 5 mL of complete RPMI into a 15 mL centrifuge tube, labeled *Target Cells.*
2. Using proper PPE, remove cells from liquid nitrogen storage and thaw cells. TIP: Be careful of contamination.
3. Quickly move vial(s) to a water bath (37°C) to thaw. While thawing, swirl 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 getting underneath the cap and into the sample.
4. When the sample is nearly thawed, remove the vial(s), 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 the 5 mL of complete RPMI in the 15 mL centrifuge tube labeled *Target Cells*. 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 the inside of the vial to recover additional thawed cells. TIP: Insert tip into complete RPMI when pipetting, be careful to not create bubbles.
7. Draw up the cell/complete RPMI mixture and pipette into the 15 mL centrifuge tube labeled *Target Cells*. TIP: Insert tip into complete RPMI when pipetting, be careful to not create bubbles.
8. Centrifuge cells for 10 minutes at 300 rcf.
9. Remove cells from centrifuge, check for cell pellet.
10. Aspirate supernatant, TIP: Be careful not to aspirate the cell pellet.
    a. Use pipette to remove last bit of supernatant.
11. Resuspend in 1 mL of fresh complete RPMI. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
12. Slowly add complete RPMI to a final density of 1 x 10^6 cells/mL.
13. Mix well 5 times with serological pipette. TIP: Be careful not to create bubbles.
14. Transfer cell suspension to flask or plate. TIP: Slowly pipette down the side of the flask as to not create bubbles.
15. 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.
16. Incubate at 37°C, 5% CO_2.
17. Passage cells every few days, depending on the requirements for specific cell type.

Chapter 3: Culture of Target Cells

Materials Required

Complete RPMI (37°C)
Incubated Cells from Chapter 2
15 mL Centrifuge Tube
Lo-Bind Microcentrifuge Tube for Cell Count
T75 Flask

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

Methods

- 1. Transfer cells from flask or plate into 15 mL centrifuge tube. TIP: Be careful not to create bubbles.
- 2. Add complete RPMI to flask or plate and rinse 5 times. TIP: Make sure to spread out the complete RPMI to gather maximum number of cells.
    - For T75 Flask add 3 mL
    - For T25 Flask add 2 mL
    - For 6 Well Plate add 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. Remove cells from centrifuge, check for cell pellet.
- 8. Aspirate supernatant. TIP: Be careful not to aspirate the cell pellet.
    a. Use pipette to remove last bit of supernatant.
- 9. Resuspend target line cells in 1 mL of complete RPMI. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
10. Slowly add complete RPMI to a final concentration of 1 x 10^6 cells/mL.
- 11. Mix with serological pipette by gently pipetting up and down 5 times. TIP: Be careful not to create bubbles.
- 12. Transfer cell suspension to flask. TIP: Slowly pipette down the side of the flask as to not create bubbles.
- 13. Spread out cell suspension by rocking flask carefully to fully cover the bottom of the flask. TIP: Be careful to not make bubbles.
14. Incubate at 37°C, 5% CO_2 and passage the target and control cells as needed over the next few days.
- CRITICAL: Passage sufficient numbers of target and control cells to perform assay at a ratio of 1:2 T cells to targets.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Chapter 4: Recovery of Cryopreserved Cells

Materials Required

Complete RPMI (37°C)
Recombinant IL-2 at 1 µg/mL (-20°C)
15 mL Centrifuge Tube
Cryopreserved PBMC
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.
3. Quickly move vial(s) to a water bath (37°C) to thaw. While thawing, swirl 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 to get underneath the cap and into the sample.
4. When the sample is nearly thawed, remove the vial(s) 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 the 5 mL of complete RPMI in the 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 the inside of the vial to recover additional thawed cells. TIP: Insert tip into complete RPMI when pipetting, be careful to not create bubbles.
- 7. Draw up the cell/complete RPMI mixture and pipette into the 15 mL centrifuge tube labeled *Thawed PBMC*. TIP: Insert tip into complete RPMI when pipetting, 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 (1µg/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. Remove cells from centrifuge, 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. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
13. Slowly add complete RPMI to a final density of 1 x 10^6 cells/mL.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

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.

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

- 16. Mix with serological pipette by gently pipetting up and down 5 times. TIP: 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 5: Post-Recovery Sample Setup

Materials Required

Complete RPMI (37°C)
15 mL Centrifuge Tube
Overnight Recovered Cells from Chapter 4 or
Fresh PBMC if Working with Fresh Samples
Lo-Bind Microcentrifuge Tube

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

Methods

- 1. Transfer cells from flask or plate into 15 mL centrifuge tube. TIP: Be careful not to create bubbles.
- 2. Add complete RPMI to flask or plate and rinse 5 times. TIP: Make sure to spread out the complete RPMI to gather maximum number of cells.
    - For T75 Flask add 3 mL
    - For T25 Flask add 2 mL
    - For 6 Well Plate add 1 mL
3. Transfer cell/complete RPMI mixture to the 15 mL centrifuge tube.
- 4. Mix with 10 mL serological pipette by gently pipetting up and down 5 times. TIP: Be careful not to create bubbles.
- 5. Take a 10 µL aliquot of 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.

7. Proceed immediately to next chapter.

Chapter 6: CD8 Sample Enrichment

Materials Required

Complete RPMI (37°C)
RoboSep Buffer (4°C)
Miltenyi CD8 Microbeads, Human, 2 mL (4°C)
MACS LS Column
Prepared Cells from Chapter 5
Enrichment Kit:
- MACS Metal Plate/Magnet Kit
- 3 x 15 mL Centrifuge Tubes (*Discard, Flow Through, CD8 Fraction*)
- Lo-Bind Microcentrifuge Tube for Post-Enrichment CD8

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

Methods

1. Remove cells from centrifuge and check for cell pellet.
2. Aspirate supernatant. TIP: Be careful not to aspirate the cell pellet.
    a. Use pipette to remove last bit of supernatant.

CRITICAL: For every 1 x 10^7 cells, resuspend in 80 µL RoboSep (4°C) and 20 µL of CD8 beads (4°C).

3. Add 80 µL of cold RoboSep to 15 mL centrifuge tube containing 1 x 10^7 or fewer cells.
4. Vortex Miltenyi CD8 Microbeads at a slow speed for 10 seconds.
5. Add 20 µL of Miltenyi CD8 MicroBeads and mix well by gently pipetting up and down 5 times.

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

6. Incubate in refrigerator (4°C) for 15 minutes. TIP: Don't incubate on ice as increased incubation times may be required.
7. After 15 minutes, add 2 mL of cold RoboSep. TIP: Not necessary to mix for this step.
8. Centrifuge cells for 10 minutes at 300 rcf.

TIP: Keep RoboSep in refrigerator during enrichment process.

9. Set up MACS sorting by setting metal plate in tissue culture hood and placing magnet on metal plate. Place LS column in magnet with wings facing out and align the 15 mL centrifuge tube labeled "Discard" under the LS column. CRITICAL: LS Column should not touch the tubes.
10. After cells are centrifuged, check for cell pellet and continue with MACS separation.
11. Aspirate RoboSep from cell pellet. TIP: Since it is a small volume, use pipette for this step to prevent accidental aspiration of the cell pellet.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

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

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

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

13. Starting 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 next step. As a reminder, be careful to not let the column dry out.

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

15. When last drop falls through to "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 next step.

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

17. Mix cell suspension by gently pipetting up and down 5 times. NOTE: This ensures that the cells are evenly dispersed after sitting.

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

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

- b. Second wash: Add 3 mL of RoboSep into LS Column after last drop passes through or does not fall. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.

- c. Third wash: Add 3 mL of RoboSep into LS Column after last drop passes through or does not fall. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.

20. After last drop of the third wash passes through or does not fall, remove the LS Column carefully from the magnet, and place carefully on the tube labeled for "CD8 fraction".

21. Cap the "Flow Through" tube. This will be used for CD4 enrichment. Do not discard.

- 22. Add 5 mL cold RoboSep to the LS column. CRITICAL: Be careful not to touch the sides.

- 23. Take plunger, smoothly push down on the plunger to push RoboSep buffer through the LS column. TIP: Lift up at the end of the plunging action so that the liquid does not splash back onto the LS Column tip.

24. Set LS column back on the "CD8 fraction" tube.

25. Loosen up plunger. Remove plunger briefly from the 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.

26. Add another 5 mL of cold RoboSep to the LS column.
- 27. Take plunger, smoothly push down on the plunger to push the RoboSep buffer through the LS Column. TIP: Lift up at the end of the plunging action so that the liquid does not splash back onto the LS Column tip.

28. Discard LS Column and plunger.
29. Centrifuge "CD8 fraction" tube and "Flow Through" (CD8 Depleted PBMC Fraction) tube for 10 minutes at 300 rcf.
30. After cells are centrifuged, check for cell pellets.
- 31. Aspirate RoboSep buffer from "CD8 fraction" and "Flow Through" (CD8 Depleted PBMC Fraction) tubes. TIP: Be careful to not aspirate cell pellet.
- 32. Use pipette to aspirate the remaining supernatant from each tube. TIP: Be careful to not aspirate cell pellet.
- 33. Add 1 mL complete RPMI to "CD8 fraction" and resuspend cell pellet. TIP: Make sure there are no clumps or bubbles.
- 34. Aliquot 10 µL of the "CD8 fraction" into a Lo-Bind Microcentrifuge tube and proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.

35. Move "CD8 fraction" tube to incubator until Cell Staining (Chapter 8).

Chapter 7: CD4 Sample Enrichment

Materials Required

Complete RPMI (37°C)
RoboSep Buffer (4°C)
Miltenyi CD4 Microbeads, Human, 2 mL (4°C)
MACS LS Column
CD8 Depleted Fraction or Overnight Recovered Cells from Chapter 5 if CD8 Depletion is Not Necessary
Enrichment Kit:
- MACS Metal Plate/Magnet Kit
- 3 x 15 mL Centrifuge Tubes (*Discard, Flow Through, CD4 fraction*)
- Lo-Bind Microcentrifuge Tube for Post-Enrichment CD4

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

Methods

- CRITICAL: For every 1 x 10^7 cells, resuspend in 80 µL RoboSep (4°C) and 20 µL of CD4 beads (4°C).

1. Add 80 µL of cold RoboSep to 15 mL centrifuge tube containing the CD8 depleted fraction of 1 x 10^7 cells or fewer.
2. Vortex the Miltenyi CD4 Microbeads at a slow speed for 10 seconds.
3. Add 20 µL of Miltenyi CD4 Microbeads and mix well by gently pipetting up and down 5 times.

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

4. Incubate in refrigerator (4°C) for 15 minutes. TIP: Don't incubate on ice as increased incubations times may be required.
5. After 15 minutes, add 2 mL of cold RoboSep. TIP: Not necessary to mix for this step.
6. Centrifuge cells for 10 minutes at 300 rcf.

- TIP: Keep RoboSep in refrigerator during enrichment process.

7. 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.
8. After cells are centrifuged, check for cell pellet and continue with MACS separation.
9. 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.
10. For 1 x 10^8 or fewer cells, resuspend with 500 µL of cold RoboSep.

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

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

11. 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 next step. As a reminder, be careful to not let the column dry out.
12. 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.
13. When last drop falls through to "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.
14. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.
15. Mix cell suspension by gently pipetting up and down 5 times. NOTE: This ensures that the cells are evenly dispersed after sitting.
16. Draw up all 500 µL of cell suspension and pipette carefully into the center of the LS column without touching sides of the column.
17. Wash 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 16. 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.

18. After the last drop of the third wash passes through or does not fall, remove the LS Column carefully from the magnet, and place carefully on the tube labeled for "CD4 fraction."
19. Cap the "Flow Through" tube and discard.
20. Add 5 mL of cold RoboSep to the LS column. CRITICAL: Be careful not to touch the sides.
21. 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.
22. Set LS Column back on the "CD4 fraction" tube.
23. 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.

24. Add another 5 mL of cold RoboSep to the LS Column.
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. Discard LS Column and plunger.
27. Centrifuge "CD4 fraction" tube for 10 minutes at 300 rcf.
28. After cells are centrifuged, check for cell pellet.
29. Aspirate RoboSep buffer from "CD4 fraction" tube. TIP: Be careful to not aspirate cell pellet.
30. Use pipette to aspirate the remaining supernatant from each tube. TIP: Be careful to not aspirate cell pellet.
31. Add 1 mL complete RPMI to "CD4 fraction" and resuspend cell pellet. Mix thoroughly by gently pipetting up and down 5 times. TIP: Make sure there are no clumps or bubbles.
32. Aliquot 10 µL of the CD4 fraction into a Lo-Bind Microcentrifuge tube and proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.
33. Move "CD4 fraction" tube to incubator until Cell Staining. (Chapter 8).

Chapter 8: Cell Staining

Materials Required

CD8 Fraction from Chapter 6
CD4 Fraction from Chapter 7
2 x Lo-Bind Microcentrifuge Tube (*CD8, CD4*)
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 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.

    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 cells from the incubator.
4. Mix CD8 cells by pipetting up and down. Transfer cells to a Lo-Bind microcentrifuge tube.
5. Repeat step 4 for CD4 cells.
6. Centrifuge cells for 10 minutes at 300 rcf.
7. After cells are centrifuged, check for cell pellets.

● 8. 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.

9. 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.

10. Centrifuge cells for 10 minutes at 300 rcf.

11. After cells are centrifuged, check for cell pellets.
- 12. 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.
13. Gently remix stain master mix.
- CRITICAL: Failure to remix stain master mix will result in poor staining.
- 14. For every 1 x 10^6 cells, add 100 µL of well mixed stain master mix to each cell suspension tube. CRITICAL: Pipet 15 times to mix the cells. Be careful to not create bubbles.
15. Incubate for 5 minutes at 37°C in the dark.
- 16. Gently pipet 15 times to mix the cell suspension. CRITICAL: Be careful to not create bubbles.
17. Incubate for an additional 5 minutes at 37°C in the dark.
- 18. After incubation, add 5 times the volume of complete RPMI. CRITICAL: Pipet 15 times to mix the cells. Be careful to not create bubbles.
19. Incubate for 10 minutes at 37°C in the dark.
20. Centrifuge the stained cells for 10 minutes at 300 rcf.
21. After cells are centrifuged, check for cell pellets.
- 22. Aspirate supernatant. TIP: Be careful not to aspirate the cell pellets.
23. Resuspend cell pellet in 500 µL of complete RPMI. Transfer CD8 cell suspension to a 15 mL centrifuge tube.
24. Repeat step for CD4 cells.
25. Take 10 µL aliquot of stained CD8 cells and stained CD4 cells and transfer to a Lo-Bind centrifuge tube for counting. Count cells using a hemocytometer and determine percent of viable cells as described in Appendix D1.
26. Centrifuge the stained cell fractions for 10 minutes at 300 rcf.
27. After cells are centrifuged, check for cell pellets.
- 28. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
29. Resuspend the cells with complete RPMI to a cell density of 1 x 10^6 cells/mL.
30. Incubate stained CD8 and CD4 cells at 37°C, 5% CO_2, until Chapter 9.

Chapter 9: Cell Stimulation

Materials Required

Complete RPMI (37°C)
15 mL Centrifuge Tube
96 Well Plate U-Bottom
Target Cell Culture(s) from Chapter 3
Bi-specific Antibody
Control Antibody
Stained CD8 Cells from Chapter 8
Stained CD4 Cells from Chapter 8
Lo-Bind Microcentrifuge Tube

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

Methods

1. Remove flask containing target cell line(s) from incubator.
2. Mix target cell culture(s) by gently pipetting up and down using a pipette. TIP: Be careful not to create bubbles.
3. Transfer target cells from flask to 15 mL centrifuge tube. TIP: Be careful not to create bubbles.
4. Add complete RPMI to flask and rinse 5 times. TIP: Make sure to spread out the complete RPMI to gather maximum number of cells.
    - For T75 Flask add 3 mL
5. Transfer target cells/complete RPMI mixture to the 15 mL centrifuge tube. TIP: Be careful not to create bubbles.
6. Take a 10 µL aliquot of target cells and transfer to Lo-Bind microcentrifuge tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
7. Centrifuge cells for 10 minutes at 300 rcf. While cells are spinning down, use hemocytometer to obtain cell counts. CRITICAL: See Appendix D1 for cell counting instructions.
8. Remove cells from centrifuge and check for cell pellet.
9. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
10. Resuspend target cells in complete RPMI to a density of 2 x 10^6 cells/mL. TIP: Resuspend as thoroughly as possible, but gently.
11. Using a P100 pipette, add 100 µL of target cells to desired number of wells in a 96 well U-bottom plate. NOTE: Concentration is 2 x 10^6 cells/mL.

CRITICAL: Use a 1:1 volume ratio for an overall density ratio of 1 T cell to 2 target cells. Volume in each well should be 200 µL.

12. Add 100 µL of the CD8+ T cells to the well(s) containing the specific target cells in the 96 well U-bottom plate. NOTE: Concentration is 1 x 10^6 cells/mL.

- a. Mix cell suspension thoroughly. TIP: Be careful not to create bubbles. Pipette thoroughly, but gently.

13. Repeat step 12 with the CD4+ T cells.
14. Add desired concentration of bispecific or control antibody to the T cell: Target cell co-cultures.

NOTE: Suggested co-culture setup:

CD4+: Target cells + CTL Abs (control)

Target cells + Bi-specific Abs (Ag-specific)

CD8+: Target cells + CTL Abs (control)

Target cells + Bi-specific Abs (Ag-specific)

15. Incubate co-culture plate for 36-48 hours at 37°C 5% CO_2.

Chapter 10: Chip Thawing

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 12).
2. Place on a 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 11: Target Cell Depletion

Materials Required

96 Well Plate Containing CD8 T Cell Co-Cultures and CD4 T Cell Co-Cultures
Sterile 1X PBS (Room Temperature)
EasySep Magnets
15 mL Centrifuge Tubes
Lo-Bind Microcentrifuge Tubes
5 mL Polystyrene Tubes
Target Cell Line Depletion Beads (4°C)

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

Methods

- CRITICAL: Target cell line depletion beads will need to be prepared prior to starting this section. See Appendix D3 for instructions. The protocol described in Appendix D3 describes how to conjugate depletion beads for two widely used target cells; K562 cells (CD235a) and Raji cells (CD19). These depletion beads can be used for any target cells expressing CD235a or CD19 respectively.

1. Resuspend CD8 T cells by pipetting up and down before transferring into a Lo-Bind Microcentrifuge Tube. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
2. Repeat step 1 using CD4 T cells.
3. Centrifuge for 10 minutes at 300 rcf.
4. Remove cells from centrifuge and check for cell pellet.
5. Aspirate supernatant with a pipette.* TIP: Be careful not to aspirate the cells.

   *NOTE: Supernatants may be stored at -80°C for bulk assay.
6. Remove deletion beads from the refrigerator (4°C) and resuspend depletion beads using a P1000 pipette. TIP: Make sure to eliminate clumps so that beads are evenly distributed among cells. Be careful not to create bubbles.

- CRITICIAL: For every 3 x 10^5 cells, add 50 µL of depletion beads (e.g., antiCD235a-conjugated beads for K562 cell depletion) to each cell pellet and return remainder of beads to 4°C.

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

7. Incubate for 10 minutes at room temperature.
8. Gently mix with a P100 pipette to ensure beads are kept in suspension. CRITICAL: Resuspend every 2 minutes to keep mixture in suspension.
9. After the incubation, add 950 µL of PBS to the CD8 T cells Lo-Bind microcentrifuge tube(s). Repeat step using CD4 T cells Lo-Bind Microcentrifuge tube(s).
10. Gently pipette up and down to resuspend. TIP: Make sure to mix well. Be careful not to create bubbles or leave clumps.
11. Transfer T cells from Lo-Bind microcentrifuge tubes to pre-labeled 5 mL polystyrene tubes.
12. Place 5 mL polystyrene tubes in an EasySep magnet for 2 minutes.
13. After 2 minutes, keep 5 mL polystyrene tubes attached to magnet and forcefully decant T cells into 15 mL centrifuge tubes. CRITICAL: Be careful not to disrupt the 5 mL polystyrene tube from magnet.
14. Remove 5 mL polystyrene tube from magnet.
15. Add 1 mL of PBS to the empty Lo-Bind microcentrifuge tube from step 11 to recover additional cells.
16. Transfer cell/PBS mixture to corresponding 5 mL polystyrene tube. Rinse the walls of 5 mL polystyrene tube well with mixture.
17. Resuspend depletion beads.

18. Place 5 mL polystyrene tubes in an EasySep magnet for 2 minutes.
19. After 2 minutes, keep 5 mL polystyrene tubes attached to magnet and forcefully decant T cells into 15 mL centrifuge tubes. CRITICAL: Be careful not to disrupt the 5 mL polystyrene tube.

    NOTE: Pool target cell depleted T cell suspensions from the same sample into one 15 mL centrifuge tube.
20. Centrifuge T cells for 10 minutes at 300 rcf.
21. Remove cells from centrifuge, check for cell pellets.
22. Aspirate supernatant. TIP: Be careful not to aspirate cell pellets.
    a. Use pipette to aspirate remainder of supernatant.
23. Gently resuspend cell pellets in 100 µL of complete RPMI.
    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.
24. Take a 10 µL aliquot and transfer to Lo-Bind microcentrifuge tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
25. Centrifuge T cells for 10 minutes at 300 rcf.
26. Remove cells from centrifuge, check for cell pellets.
27. Aspirate supernatant. TIP: Be careful not to aspirate cell pellets.
    a. Use pipette to aspirate remainder of supernatant.
28. Gently resuspend T cells to a cell density of 1 x 10^6 cells/mL in complete RPMI. Proceed to Chapter 12.

Chapter 12: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 10
CD8 T Cells at 1 x 10^6 Cells/mL
CD4 T 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
2. Resuspend CD8 T cells by pipetting up and down. Pipette 30 µL of cell suspension into IsoCode chip. CRITICAL: Be careful not to create bubbles. Insert pipette tip vertically into inlet port until tip lightly touches bottom, and slowly pipette 30 µL into inlet port. Be careful not to eject second step of pipette–it will cause bubbles.
3. Resuspend CD4 T cells by pipetting up and down. Pipette 30 µL of cell suspension into IsoCode chip. CRITICAL: Be careful not to create bubbles. Insert pipette tip vertically into inlet port until tip lightly touches

- bottom, and slowly pipette 30 µL into inlet port. Be careful not to eject second step of pipette–it will cause bubbles.

4. Let IsoCode chip 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 to your instrument's loading instructions for details.

D: Appendix

D1 Protocol: Cell Quantification & Viability

Materials Required

Hemocytometer
10 µL aliquot of cells
Trypan Blue

NOTE: Automated cell counters can be used in this protocol only prior to Chapter 8 due to spectral overlap of the stains. Manual cell counting is required after Chapter 8.

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 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 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 $3x10^6$)
15 mL Centrifuge Tubes
Lo-Bind Microcentrifuge Tube(s)
Ficoll Paque

- CRITICAL: It is recommended to start this protocol with a minimum of $3x10^6$ total cells.

1. Carefully add 6 mL of Ficoll to the bottom of the required amount 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 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 VERY SLOWLY to the tubes 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 break 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 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.
12. Transfer cells into the prepared 15 mL centrifuge tube(s) containing complete RPMI.

- 13. Take 10 µL of cell/RPMI mixture(s) and transfer into Lo-Bind microcentrifuge tube(s). Proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

D3 Protocol: Bulk Depletion Bead Preparation

Materials Required

Dynabeads M-280 Streptavidin (4°C)
Biotin Anti-Human CD235a **or** Biotin Anti-Human CD19 Antibody (4°C)
Sterile 1X PBS (Room Temperature)
0.1% BSA (Room Temperature)
EasySep Magnet
1 x 15 mL Centrifuge Tube
1 x 5 mL Polystyrene Tube

NOTE: This protocol produces 2 mL of conjugated depletion beads.

Table 11: Antibodies Used for Specific Target Cell Lines

Target Cell Line	Antibody Needed
K562	Biotin Anti-Human CD235a
Raji	Biotin Anti-Human CD19

1. Remove Dynabeads M-280 from 4°C.
2. Using a P1000, resuspend Dynabeads M-280 well. **NOTE: Dynabeads M-280 will sit pelleted at the bottom of the container. Resuspension should achieve a solid-colored mixture.**
3. Pipette 500 µL of beads into a 5 mL polystyrene tube. Return any remaining Dynabeads to 4°C for storage according to manufacturer's protocol.
4. Pipette 1 mL of PBS into the 5 mL polystyrene tube and gently mix up and down 3-4 times.

   NOTE: Mixing removes any remaining sodium azide from the manufacturer's storage buffer.
5. Place the 5 mL polystyrene tube in an EasySep Magnet for 1 minute.
6. Gently decant supernatant into a 15 mL centrifuge tube. **CRITICAL: Do not disturb the polystyrene tube in magnet. Doing so will transfer beads into decanted supernatant.**
7. Remove the 5 mL polystyrene tube from EasySep Magnet.
8. Repeat steps 4-7 one more time for a total of two washes.
9. Pipette 100 µL of antibody into the 5 mL polystyrene tube. Return any remaining antibody to 4°C for storage according to manufacturer's protocol. **NOTE: Type of antibody (CD235a or CD19) will be dependent on target cell line. Refer to Table 11.**
10. Mix well to ensure antibody and beads are in suspension by gently pipetting up and down 3-4 times.
11. Incubate 5 mL polystyrene tube for 30 minutes at room temperature in the dark.
    a. With a P100, pipette mixture every 5 minutes to keep beads in suspension.
12. After 30 minutes, add 1 mL of 0.1% BSA to 5 mL polystyrene tube and mix by gently pipetting up and down 3-4 times.
13. Place the 5 mL polystyrene tube in an EasySep Magnet for 1 minute.

14. Gently decant supernatant into a 15 mL centrifuge tube. CRITICAL: Do not disturb the polystyrene tube in magnet. Doing so will transfer beads into decanted supernatant.
15. Remove the 5 mL polystyrene tube from EasySep Magnet.
16. Add 1 mL of 0.1% BSA to the 5 mL polystyrene tube, making sure to rinse the beads from the side of the tube, and mix by gently pipetting up and down 3-4 times.
17. Place the 5 mL polystyrene tube in an EasySep Magnet for 1 minute.
18. Gently decant supernatant into a 15 mL centrifuge tube. CRITICAL: Do not disturb the polystyrene tube in magnet. Doing so will transfer beads into decanted supernatant.
19. Repeat steps 15-18 three more times for a total of five washes.
20. Resuspend conjugated beads in 2 mL of 0.1% BSA.
21. Seal the 5 mL polystyrene tube by capping the tube and tightly wrapping the capped tube with parafilm.
22. Store conjugated bead mixture upright in the 5 mL polystyrene tube at 4°C.

    NOTE: Conjugated depletion beads can be stored at 4°C for up to three months.

D4 Protocol: Test Depletion Beads (Optional)

Materials Required

Anti-Human CD235a or Anti-Human CD19 Conjugated Depletion Beads (4°C)
T Cells
Target Cells
96 Well Plate U-Bottom
15 mL Centrifuge Tube
Lo-Bind Microcentrifuge Tubes

NOTE: It is recommended to complete this section to demonstrate the efficiency of depletion beads produced according to the protocol in Appendix D3 before proceeding to your experiment.

NOTE: T Cells used in this Appendix could be the same cells that are intended for use with this protocol or healthy control cells. It is recommended to test the same cell subset that will be run in the protocol. For instance, if you intend to assay CD8+ T cells on the Bruker instrument then it is recommended to use CD8+ T cells in this depletion bead test.

Before starting this test, obtain an enriched T cell population. Refer to Chapters 6 and 7 for instructions. Ensure enriched T cell population is suspended at 1 x 10^6 cells/mL prior to starting this protocol. Additionally, ensure target cells are available for this test. Refer to Chapters 2 and 3 for instructions.

1. Remove target cell line(s) from the incubator.
2. Mix target cell line(s) gently up and down using a pipette. TIP: Be careful not to create bubbles.
3. Transfer target cells from flask into 15 mL Centrifuge Tube. TIP: Be careful not to create bubbles.

- 4. Add complete RPMI to flask and rinse 5 times. TIP: Make sure to spread out the complete RPMI to gather maximum number of cells.
    - For T75 Flask add 3 mL
- 5. Transfer target cells/complete RPMI mixture to the 15 mL Centrifuge Tube. TIP: Be careful not to create bubbles.
- 6. Take a 10 µL aliquot of target cells and transfer to a Lo-Bind Microcentrifuge Tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
- 7. Centrifuge cells for 10 minutes at 300 rcf. While cells are spinning down, use hemocytometer to get cell counts. 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.

8. Remove cells from centrifuge and check for cell pellet.

- 9. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
- 10. Resuspend target cells with complete RPMI to a cell concentration of 2 x 10^6 cells/mL. TIP: Resuspend as thoroughly as possible, but gently.

Table 12: Test Conditions for Bulk Depletion Beads

Condition	To be Depleted?	Expectation for Test
Target Cells Alone	Yes	>80% of target cells depleted
Target Cells & T Cells	Yes	>80% of target cells depleted Low depletion of T cells, cannot exceed 50%
Target Cells & T Cells	No	Both target cells and T cells remain
T Cells Alone	Yes	Low depletion of T cells, cannot exceed 50%

11. Using a P100 pipette, add 100 µL of target cells to desired number of wells on the 96 well U-bottom plate. NOTE: Concentration is 2 x 10^6 cells/mL. At minimum, 1 well should be plated for each target cell containing condition (minimum of 3 wells).
12. Using a P100 pipette, add 100 µL of pre-enriched T cells to desired number of wells containing target cells in the 96 well U-bottom plate. NOTE: Concentration is 1 x 10^6 cells/mL. Total volume in each well will be 200 µL.

- CRITICAL: Do not add T cells to at least one well on the 96 well U-bottom plate. The well(s) will serve as the target cells alone condition.

13. Using a P100 pipetted, add 100 µL of pre-enriched T cells to desired number of **empty** wells on the 96 well U-bottom plate. **NOTE: Concentration is 1 x 10^6 cells/mL**.

- 14. Mix all cell suspensions thoroughly. **TIP: Be careful not to create bubbles. Pipette thoroughly, but gently.**

15. Incubate 96 Well Plate U-Bottom for 20 hours at 37°C, 5% CO_2. **NOTE: 1 hour incubation can be used as an approximation if needed.**

16. Deplete cells for the conditions outlined in Table 12. Refer to Chapter 11 steps 1 through 23.

- 17. After depletion, take a 10 µL aliquot of each of the 4 conditions and transfer to a Lo-Bind microcentrifuge tube for cell counting and visual interpretation. **CRITICAL: See Appendix D1 for cell counting instructions. Cell counting must be completed manually using a hemocytometer to allow for visual inspection of each condition. Target cell type dependent, cell size or cell morphology can be used to distinguish between the target cells and the T cells.**

18. Refer to Table 12 for expectations.

    For assistance in optimizing your depletion beads or any other steps in the assay, please contact your local Field Applications Scientist or the Scientific Support team. Contact information for the Scientific Support team can be found in the Troubleshooting and References section.

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	• Use appropriate dilutions recommended in **Appendix D1** • Do a recount if initial count does not seem accurate • Quick spin Trypan Blue to pellet potential debris, remove aliquot from top of Trypan Blue. Start with fresh aliquot of Trypan Blue. • Count within 15 minutes of staining cells
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 8** • Use only freshly prepared cell stain 405 per **Chapter 8** • Ensure all media is removed from cell pellet in **step 8.8**

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 **12.2** 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.
		• Closely follow the Critical steps and tips in **Chapter 6 and 7** (CD8, CD4 Sample Enrichment)
		• Load recommended number of cells (30,000 cells per chip) (**Chapter 12**)
		• 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 • Low viable cells due to low viability input sample and lack of utilization of Ficoll Paque • 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 4** to ensure protocol is being performed with the highest quality of cells. Use Ficoll Paque in **Appendix D2** if viability is less than 80% • 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 T Cell to target cell ratio was not used • Recommended incubation duration was not used	• Use recommended T Cell to target cell ratio as listed in **steps 9.10 and 9.11** • Use incubation timing listed in **Chapter 9**