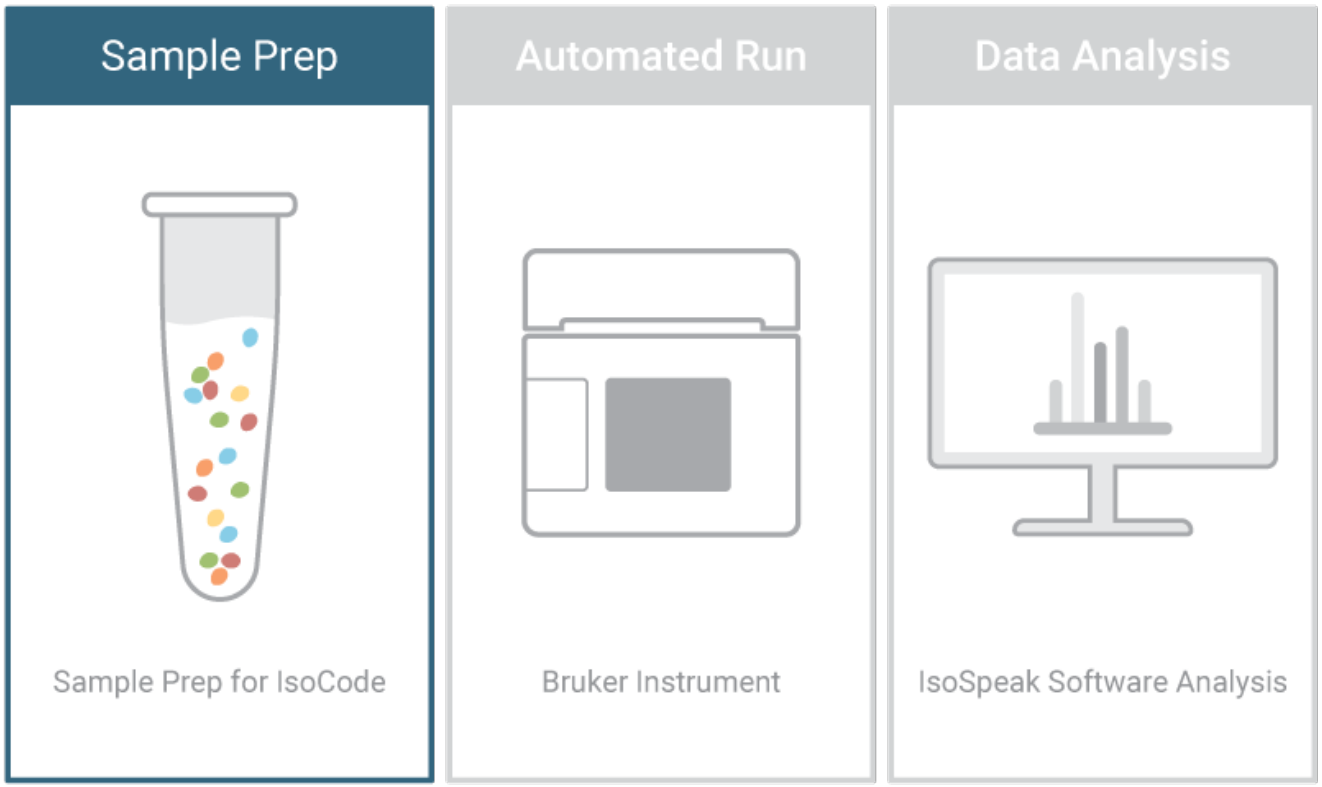
IsoCode Single-Cell Adaptive Immune: Human PBMC with CD4 and CD8 Subsets Combined on a Single Chip Protocol

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 Stimulation of T cells for 24 hours with CD3/CD28 or for 1.5 hours with PMA/ionomycin. Staining and Loading of T cells, stimulated with PMA/ionomycin, onto IsoCode chip.

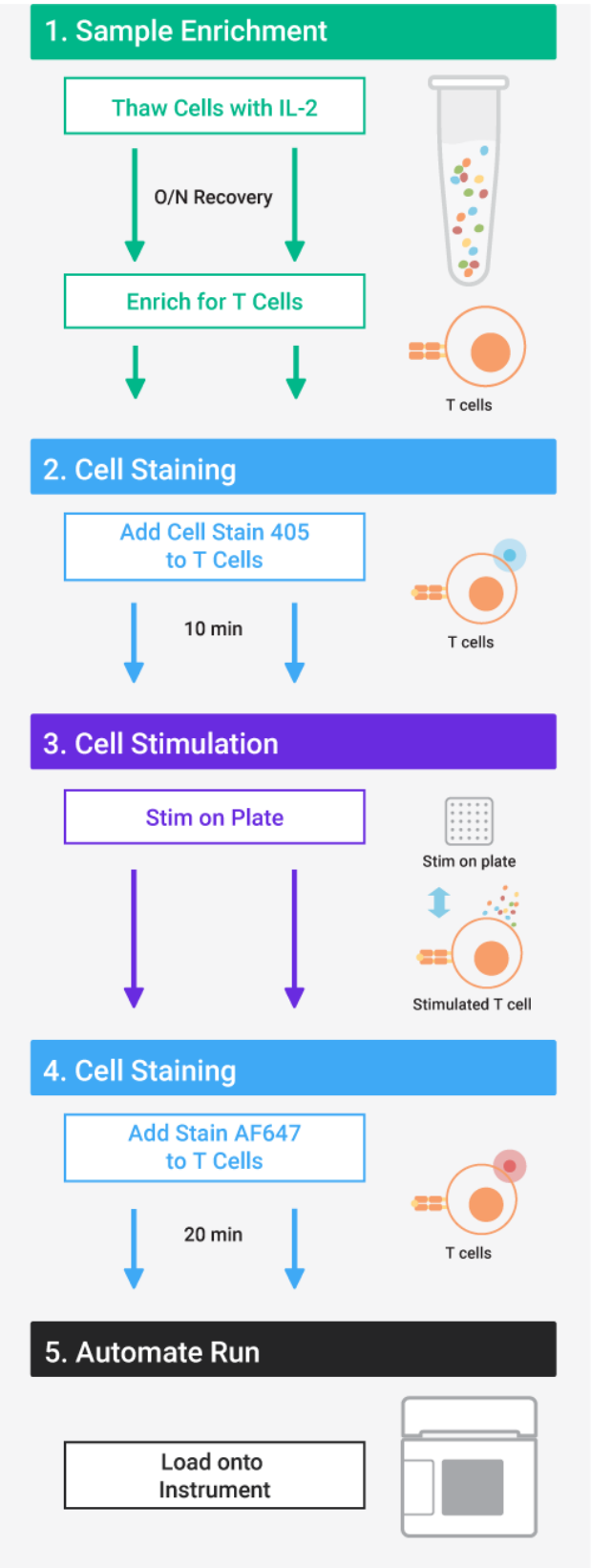
Day 3: Staining and Loading of T cells, stimulated with CD3/CD28, onto IsoCode chip.

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. Stimulation time may vary and is dependent upon the experimental design and/or cells.

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:
When loading CD4 and CD8 cells onto the same chip, it is critical to understand the CD4 and CD8 ratio of a sample. The CD4 and CD8 ratio may not be equal and can impact the number of quality cells for each cell subset. If a high number of quality cells for either cell subset is required, please utilize an alternative protocol where the subsets are enriched and loaded individually onto separate IsoCode chips.

NOTE:
In IsoSpeak, please note the presence or absence of AF647 will determine the cell subset. The CD8 T cell fraction is positive for both cell stain 405 and AF647. The presumptive CD4 T cell fraction is positive for cell stain 405 and negative for AF647.



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Safety Warnings

- Read MSDS documents of all materials prior to use.

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

Required Reagents, Consumables and Equipment

Table 1: Required 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
- Alexa Fluor 647 anti-human CD8 stain (AF647-CD8) [ordered separately]

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
- Alexa Fluor 647 anti-human CD8 stain (AF647-CD8) [ordered separately]

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
T25 Flask	N/A	Corning	353108
T75 Flask	N/A	Corning	430641U
96 Well Plate Flat Bottom	N/A	Corning	353072
MACS LS Column	N/A	Miltenyi	130-042-401
Centrifuge Tubes*	Polypropylene, 15 mL	VWR	CA62406-200
Lo-Bind Microcentrifuge Tubes, Sterile	1.5 mL	USA Scientific	4043-1081
Pipette Tips (Filtered)	10 µL Graduated Filter Tips 100 µL Graduated Filter Tips 1000 µL XL Graduated Filter Tips	USA Scientific	1181-3710 1183-1740 1182-1730
Serological Pipette	2 mL Pipette 5 mL Pipette 10 mL Pipette	USA Scientific	1072-0510 1075-0110 1071-0810
Syringe with BD Luer-Lok Tip	10 mL	VWR	75846-756
0.2 µm Syringe Filter with Acrylic Housing	N/A	VWR	28145-501
Fisherbrand Disposable PES Filter Units (0.20 µm)	500 mL	Fisher Scientific	FB12566504

*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 Pan T Cell Isolation Kit, Human	N/A	Miltenyi	130-096-535
Trypan Blue	0.4%	Gibco	15250-061
Cell Activation Cocktail (without Brefeldin A)	500x	BioLegend	423301
CD3 Monoclonal Antibody (OKT3), Functional Grade	1 mg/mL	ThermoFisher/Invitrogen	16-0037-85
CD28 Monoclonal Antibody (CD28.2), Functional Grade	1 mg/mL	ThermoFisher/Invitrogen	16-0289-81
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
Alexa Fluor 647 anti-human CD8 (AF647-CD8)	STAIN-1003-1	Red

Table 5: Required Equipment

Equipment	Source	Catalog Number
IsoLight, IsoSpark, or IsoSpark Duo Instrument	Bruker	ISOLIGHT-1000-1, ISOSPARK-1000-1, or ISOSPARK-1001-1
Culture Hemocytometer	(Fisher) Hauser Levy	02-671-55A
Hemocytometer Cover Glass	(Fisher) Hauser Levy	02-671-53
MidiMACS Separator	Miltenyi	130-042-302
MACS MultiStand	Miltenyi	130-042-303

Table 6: General Equipment

Equipment	Requirements
Pipette	P10, P100, P200, P1000
Pipettor	Ability to pipette between 1 and 10 mL
Incubator	37°C, 5% CO_2
Tabletop Centrifuge	Temperature controlled*; swinging bucket rotor; ability to centrifuge 15 mL conical tubes
Microcentrifuge	Temperature controlled*; fixed rotor; ability to centrifuge 1.5 mL microcentrifuge tubes
Mini centrifuge	Ability to spin micro sample sizes
Water Bath	Ability to heat to 37°C
Microscope	Inverted light microscope with 10x and 20x objectives
Vortex Mixer	Ability to vortex vials and microcentrifuge tubes; adjustable speed

*Temperature controlled centrifuges are required so that centrifuging steps can be conducted at room temperature without risk of overheating. Temperature on centrifuges should be set to 21°C.

B. Before Getting Started

1. Important Precautions

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 (1XPBS) without Calcium or Magnesium	1X	1X	99 mL initially*	Gibco/10010072

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

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

Ingredient	Stock Concentration	Final Concentration	Amount for 10 mL	Vendor/Catalog
1% BSA in 1X PBS (sterile filtered)	1%	1%	9.95 mL	Table 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.

Additional Reagents to Be Prepared

NOTE: Cell Activation Cocktail (without Brefeldin A) is a premix of 40.5 µM PMA and 669.3 µM ionomycin. Thaw Cell Activation Cocktail (without Brefeldin A) and prepare 15 µL aliquots. Store aliquots at -70°C for no longer than 1 year. 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
- Alexa Fluor 647 anti-human CD8 stain (AF647-CD8) [ordered separately]

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
- Alexa Fluor 647 anti-human CD8 stain (AF647-CD8) [ordered separately]

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: Coat Culture Plates with anti-CD3 (Optional)

Materials Required

Sterile 1X PBS (Room Temperature)
Anti-CD3 (4°C) 10 µg/mL
15 mL Centrifuge Tube (*anti-CD3 Dilution*)
96 Well Plate Flat Bottom (*PBMC, Date, Time*)

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

Methods

NOTE: There are two stimulation options for this protocol: CD3/CD28 stimulation or PMA/ionomycin stimulation. If your experimental design utilizes CD3/CD28, complete this chapter. Otherwise, proceed immediately to Chapter 3.

- 1. Vortex the anti-CD3 tube at a slow speed for 10 seconds. TIP: Ensure contents are well suspended.
- 2. Spin anti-CD3 in mini centrifuge for 10 seconds. TIP: Ensure that contents are all in the bottom of the vial.

3. Preparation of anti-CD3 dilution in 1X PBS to a final concentration of 10 µg/mL in a 15 mL centrifuge tube:
    a. Dilute 20 µL of 1 mg/mL anti-CD3 into 2 mL of 1X PBS.
    b. Use 1 mL pipette to mix.
4. Pipette 100 µL of anti-CD3 dilution into 20 wells on a 96 well flat bottom plate. Wells coated with anti-CD3 dilution will be used for stimulation in Chapter 7. Leave the remaining wells empty for the unstimulated condition. NOTE: It is recommended to coat at least 2 wells per sample being run. Scale up the amount of anti-CD3 dilution if more than 20 coated wells are required. Note which wells have been coated.

- CRITICAL: Be careful not to create air bubbles.

5. Incubate covered plate at 4°C overnight.

Chapter 3: 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 complete RPMI in 15 mL centrifuge tube, labeled *Thawed PBMC*. TIP: Insert tip into complete RPMI when pipetting, be careful to not create bubbles.

- 6. Take 1 mL of complete RPMI and pipette into original thawed cell vial. Rinse inside the vial with the complete RPMI to recover additional thawed cells. TIP: Insert tip into complete RPMI, be careful to not create bubbles.
- 7. Draw up cell/complete RPMI mixture and pipette into the 15 mL centrifuge tube, labeled *Thawed PBMC*. TIP: Insert tip into complete RPMI and pipette gently up and down. Be careful to not create bubbles.

8. Centrifuge cells for 10 minutes at 300 rcf.

9. While the cells are centrifuging, take the IL-2 (1 µg/mL) out from -20°C and thaw at room temperature.

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

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

- 11. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.

    a. Use pipette to remove last bit of supernatant.

12. Resuspend cell pellet in 1 mL of fresh complete RPMI.

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

13. Slowly add additional complete RPMI to a final concentration of 1 x 10^6 cells/mL.

14. Mix thawed IL-2 thoroughly by carefully pipetting up and down.

15. Dilute 100 µL of 1 µg/mL IL-2 per 10 mL of cell suspension to a final concentration of 10 ng/mL IL-2.

- CRITICAL: Discard thawed IL-2 aliquot if there is any volume remaining. IL-2 must only be thawed once.
- 16. Mix with serological pipette. TIP: Gently pipet up and down 3-5 times, be careful to not create bubbles.
- 17. Transfer cell suspension to flask or plate. TIP: Slowly pipette down the side of the flask as to not create bubbles.
- 18. Spread out cell suspension by rocking flask or plate carefully to fully cover the bottom of the container. TIP: Be careful to not make bubbles.

19. Move to incubator for overnight recovery at 37°C, 5% CO_2. NOTE: The time period for overnight recovery is considered 16 – 20 hours, but not exceeding 24 hours.

Chapter 4: Post-Recovery Sample Setup

Materials Required

Complete RPMI (37°C)
15 mL Centrifuge Tube
Overnight Recovered Cells from Chapter 3 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 15 mL centrifuge tube.
- 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.
- CRITICAL: If cells are less than 80% viable, proceed to Appendix D2 Dead Cell Depletion Protocol using Ficoll.
7. Proceed immediately to next chapter.

Key: ● TIP, ● CRITICAL, ●OPTIONAL

Chapter 5: Pan T Sample Enrichment

Materials Required

RoboSep Buffer (4°C)
Miltenyi Pan T Cell Isolation Kit, Human:
    1 mL Pan T Cell Biotin-Antibody Cocktail, Human (4°C)
    2 mL Pan T Cell MicroBead Cocktail, Human (4°C)
MACS LS Column
Prepared Cells from Chapter 4
Enrichment Kit:
    MACS Metal Plate/Magnet Kit
    2 x 15 mL Centrifuge Tubes (*Discard, Flow Through*)
    Lo-Bind Microcentrifuge Tube for Post-Enrichment T Cells

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

Methods

1. Remove the centrifuged cells and check for cell pellet.
2. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
- CRITICAL: For every 1 x 10^7 cells, resuspend in 40 µL RoboSep (4°C) and 10 µL of Pan T Cell Biotin-Antibody Cocktail (4°C).

3. Add 40 µL of cold RoboSep to 15 mL centrifuge tube containing 1 x 10^7 or fewer cells.
4. Add 10 µL of Miltenyi Pan T Cell Biotin-Antibody Cocktail and mix well by gently pipetting up and down 5 times.
5. Incubate in refrigerator (4°C) for 5 minutes. TIP: Don't incubate on ice as increased incubation times may be required.

- CRITICAL: For every 1 x 10^7 cells, resuspend in 30 µL RoboSep (4°C) and 20 µL of Pan T Cell MicroBead Cocktail (4°C).

6. Add 30 µL of cold RoboSep to 15 mL centrifuge tube containing 1 x 10^7 or fewer cells.
7. Add 20 µL of Miltenyi Pan T Cell Microbead Cocktail and mix well by gently pipetting up and down 5 times.

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

8. Incubate in refrigerator (4°C) for 10 minutes. TIP: Don't incubate on ice as increased incubation times may be required.

- 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. Add 400 µL of cold RoboSep to cell suspension for 1 x 10^7 or fewer cells. TIP: For 2 x 10^7 cells, add 300 µL instead of 400 µL of cold RoboSep to the cell suspension. 500 µL is the minimum volume required for loading LS column for up to 1 x 10^8 cells.
    a. Mix well 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 the 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 the last drop falls through to the "Discard" tube, move the rack over so the LS column is over the "Flow Through" tube. CRITICAL: Be careful not to let column dry. If there is one drop remaining that will not fall, move on to the next step.
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 column with 3 mL of cold RoboSep.
    a. Rinse inside walls of cell suspension tube with 3 mL of cold RoboSep before transferring the mixture to LS Column. NOTE: This is to retrieve any cells that have been left behind.
        i. Pipette all the mixture into LS Column after last drop passes through or does not fall from step 16. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.
18. After the last drop of the wash passes through or does not fall, cap the "Flow Through" tube, this fraction represents the enriched T Cells.
19. Discard LS Column.
20. Mix cell suspension well by pipetting up and down 5 times. TIP: Be careful not to create bubbles.
21. 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.
22. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells and determine percent of viable cells. CRITICAL: See Appendix D1 for cell counting instructions.
23. Proceed immediately to next chapter.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Chapter 6: Cell Staining (Violet)

Materials Required

T Cell Fraction from Chapter 5
2 x Lo-Bind Microcentrifuge Tubes (*Stain Master Mix*, *T Cell*)
2 x 15 mL Centrifuge Tubes (*T Cell – Unstim*, *T Cell – Stim*)
Sterile 1X PBS (Room Temperature)
Complete RPMI (37°C)
Cell Stain 405 (-20°C)
Cell Stain 405 Diluent (DMSO) (-20°C)

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

Methods

1. Prepare cell stain 405 stock.
    a. Thaw tube of cell stain 405 diluent (DMSO) at room temperature.
    b. Spin tubes of cell stain 405 and cell stain 405 diluent (DMSO) in a mini centrifuge for 10 seconds to collect the contents at the bottom of the tubes.
    c. Add 20 µL of cell stain 405 diluent (DMSO) directly to the tube of cell stain 405. Pipet up and down 15 times gently to resuspend.

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

● 2. Prepare stain master mix by diluting 2 µL of cell stain 405 into 1 mL of 1X PBS in a Lo-Bind microcentrifuge tube (1:500 final dilution). With the same pipette tip, pipette up and down 10 times to ensure all cell stain 405 has been released. Depending on sample number and cell count, additional tubes of stain master mix may need to be prepared. CRITICAL: Failure to follow these steps will negatively impact cell counts.
    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 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 supernatant from the cell pellet. TIP: Be careful not to aspirate the cell pellets.

5. Gently remix stain master mix.

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

● 6. For every 1 x 10^6 cells, add 100 µL of **well mixed** stain master mix to each cell suspension tube. CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.

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 5 times the 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. Take 10 µL of cells to count. Count cells using a hemocytometer and determine percent of viable cells as described in Appendix D1. TIP: Cell counting can be done while cells are incubating.
13. After incubation, split the cell suspension in half into two new centrifuge tubes.
    a. Pipette one half of the cell suspension into a 15 mL centrifuge tube labeled "T Cells - Unstim".
    b. Pipette the other half of the cell suspension into a 15 mL centrifuge tube labeled "T Cells – Stim".
14. Centrifuge stained cells for 10 minutes at 300 rcf.
15. Proceed immediately to next chapter.

Chapter 7: Cell Stimulation

NOTE: Please read before proceeding with cell stimulation.

There are 2 options for cell stimulation depending on your experimental design:

1. Cell stimulation with Cell Activation Cocktail described in Chapter 7a
2. Cell stimulation with CD3 and CD28 as described in Chapter 7b.

Please contact your Field Application Scientist with any questions on your experimental design.

Chapter 7a: Cell Activation Cocktail Stimulation

Materials Required

Complete RPMI (37°C)
Cell Activation Cocktail (without Brefeldin A) (-70°C)
Stained T Cells in Two Tubes from Chapter 6
96 Well Flat Bottom Plate
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. Retrieve the single use aliquot of Cell Activation Cocktail (without Brefeldin A) from -70°C.
2. After the Cell Activation Cocktail (without Brefeldin A) is completely thawed, spin down in a mini centrifuge for 10 seconds to collect the contents at the bottom of the tubes. TIP: Ensure that contents are all in the bottom of the vial.

3. Prepare Complete RPMI Unstimulated media.
    a. Aliquot 5 mL complete RPMI into a 15 mL centrifuge tube labeled "Complete RPMI Unstimulated" — set this complete RPMI aside as it will serve as the complete RPMI used for the unstimulated (negative control) condition.

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

● 4. Prepare Cell Activation Cocktail complete RPMI mixture. Aliquot 5 mL complete RPMI into a 15 mL centrifuge tube labeled "Cell Activation Cocktail Complete RPMI." CRITICAL: Volume required is dependent on number of cells.
    a. Aliquot 5 mL complete RPMI into a 15 mL centrifuge tube labeled "Cell Activation Cocktail Complete RPMI."
    b. Add 10 µL of Cell Activation Cocktail (without Brefeldin A) to the complete RPMI.
    c. Use serological pipette to mix thoroughly.

● CRITICAL: Discard remaining/unused Cell Activation Cocktail (without Brefeldin A)—they are single use only and cannot be refrozen.

5. Remove the centrifuged cells and check for cell pellets.

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

● 7. Using the complete RPMI set aside for the unstimulated condition, resuspend T cells in the 15 mL centrifuge tube labeled "T Cells - Unstim" to a cell concentration of 1 x 10^6 cells/mL. TIP: This step is for preparing the unstimulated (negative control) cells. This complete RPMI is not supplemented with any stimulants.

8. Use a pipette to mix the Cell Activation Cocktail stimulation complete RPMI mixture to ensure it is evenly distributed.

● 9. Using the Cell Activation Cocktail stimulation complete RPMI mixture from step 8, resuspend T cells in the 15 mL centrifuge tube labeled "T Cells – Stim" to a cell concentration of 1 x 10^6 cells/mL. TIP: Resuspend as thoroughly as possible, but gently.

● 10. Plate 100 µL of the unstimulated T cells, per well, on the 96 well plate flat bottom. TIP: Plate at least two wells on the 96 well plate flat bottom for the unstimulated condition.

● 11. Mix "T cells – Stim" by pipetting up and down gently 5 times. Add 100 µL of cell suspension to empty wells on the 96 well plate flat bottom. CRITICAL: Be careful not to create bubbles. This will maximize even stimulation of cell suspension.

12. Incubate plate for 1.5 hours at 37°C, 5% CO_2.

Chapter 7b: CD3 and CD28 Stimulation

Materials Required

Complete RPMI (37°C)
Anti-CD28 (4°C)
Sterile 1X PBS (Room Temperature)
Prepared anti-CD3 Coated 96 Well Plate from Chapter 2
15 mL Centrifuge Tubes
Stained T Cells in Two Tubes from Chapter 6

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) condition.

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

- 2. Vortex anti-CD28 at a slow speed for 10 seconds. TIP: Ensure contents are well suspended.
- 3. Spin anti-CD28 in a mini centrifuge for 10 seconds. TIP: Ensure that contents are all in the bottom of the vial.
- 4. Prepare anti-CD28 and complete RPMI mixture. Supplement complete RPMI with 5 µg/mL of anti-CD28 into a 15 mL centrifuge tube labeled "CD28 Complete RPMI". CRITICAL: Volume is dependent on number of cells.
    a. Dilute 25 µL of 1 mg/mL anti-CD28 into 5 mL of complete RPMI in a 15 mL centrifuge tube labeled "CD28 Complete RPMI".
    b. Use serological pipette to mix thoroughly.
5. Remove the centrifuged cells and check for cell pellets.
- 6. Aspirate supernatant with pipette. TIP: Make sure to use a manual pipette to prevent accidental aspiration of cell pellet.
- 7. Using the complete RPMI set aside for the unstimulated condition, resuspend T cells in the 15 mL centrifuge tube labeled "T Cells – Unstim" to a cell concentration of 1 x 10^6 cells/mL. TIP: This step is for preparing the unstimulated (negative control) cells. This complete RPMI is not supplemented with any stimulants.
8. Use a serological pipette to mix the anti-CD28 and complete RPMI mixture to ensure it is evenly distributed.
- 9. Using the anti-CD28/complete RPMI mixture from step 8, resuspend T cells in the 15 mL centrifuge tube labeled "T Cells – Stim" to a cell concentration of 1 x 10^6 cells/mL. TIP: Resuspend as thoroughly as possible, but gently.

10. Prepare plate.
    a. Remove previously prepared anti-CD3-coated 96 well plate from refrigerator.
    b. Aspirate the anti-CD3 dilution from 96 well plate. TIP: Aspirate from the edge of well.
    c. Add 100 µL of 1X PBS to each well previously coated with anti-CD3 to rinse.
    d. Aspirate 1X PBS from each well previously coated with anti-CD3. TIP: Aspirate from the edge of the well.
11. Mix "T cells – Stim" by pipetting up and down gently 5 times. Add 100 µL of cell suspension to coated wells on plate. CRITICAL: Be careful not to create bubbles. This will maximize even stimulation of cell suspension.
12. Mix "T cells – Unstim" by pipetting up and down gently 5 times. Plate 100 µL of the unstimulated T cells, per uncoated well, on the 96 well plate flat bottom. TIP: Plate at least two wells on the 96 well plate flat bottom for the unstimulated condition.
13. Incubate plate for 24 hours at 37°C, 5% CO_2.

Chapter 8: 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 10).
2. Place on bench to thaw at ambient temperature 30 - 60 minutes prior to use.
3. While chips thaw, prepare liquid reagents and setup in the Bruker instrument. Refer to your instrument's system guide for detailed instructions.

Chapter 9: Surface Marker-Specific Staining (Red)

Materials Required

Stimulated & Unstimulated T Cells in 96 Well Plate from Chapter 7
2 x Lo-Bind Microcentrifuge Tubes (*Unstim, Stim*)
Complete RPMI (37°C)
AF647 anti-human CD8 (4°C)

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

Methods

1. Remove 96 well plate with T cells from incubator.
2. Mix T cells by pipetting up and down. Transfer cells to a Lo-Bind microcentrifuge tube by using P100 pipette to draw up 100 µL at a time in a gentle, circular motion until well is empty. NOTE: Pool wells if there are replicates.
3. Take a 10 µL aliquot of your cells and transfer to a Lo-Bind microcentrifuge tube for cell counting.
4. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells and determine percent of viable cells as described in Appendix D1.
5. After cells are centrifuged, check for cell pellets.
6. Aspirate supernatant with a pipette.* TIP: Be careful not to aspirate the cell pellets.

   *NOTE: Supernatants may be stored at -80°C for bulk assay.
7. Spin tube of AF647 anti-human CD8 in a mini centrifuge for 10 seconds to collect stain at the bottom of the tube.

CRITICAL: For every 1 x 10^5 cells, resuspend in 18 µL of complete RPMI and 2 µL of surface marker stain.

8. Resuspend cell pellets with 18 µL complete RPMI for 1 x 10^5 or fewer cells.
9. Add 2 µL of AF647 anti-human CD8 to T cell samples (1:10 final dilution) for 1 x 10^5 or fewer cells. Mix gently by pipetting up and down.
10. Incubate for 20 minutes at room temperature in the dark.
11. After incubation, add 1 mL of complete RPMI to each sample tube. TIP: Mix gently, be careful not to create bubbles.
12. Centrifuge stained cells for 10 minutes at 300 rcf.
13. After cells are centrifuged, check for cell pellets.
14. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
15. Resuspend the cells with complete RPMI to a cell density of 1 x 10^6 cells/mL. Proceed to Chapter 10.

Chapter 10: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 8 Unstimulated T Cells at 1 x 10^6 cells/mL Stimulated 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 of chip.
- 2. Resuspend T cell 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. Let IsoCode chips sit for one minute on a flat surface.
- 4. Check bottom of chip to ensure liquid has entered the chip. TIP: If liquid has not flowed, tap IsoCode chip on flat surface lightly.
5. When inserting IsoCode chip into the instrument, make sure the logo is facing up and towards you with the magnet facing the instrument. Take the blue film off while inserting each IsoCode chip into the instrument.

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

D. Appendix

D1 Protocol: Cell Quantification & Viability

Materials Required

Hemocytometer
10 µL aliquot of cells
Trypan Blue

NOTE: Automated cell counters can be used in this protocol prior to Chapter 6 due to spectral overlap of the stains. Manual cell counting is required after Chapter 6.

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

1. Quick spin the Trypan Blue to pellet potential debris. Remove aliquot from the top of Trypan Blue.
2. Using a P10 pipette, add equal volume of Trypan blue solution to 10 µL of sample. Mix gently to resuspend.
   TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
3. Load onto hemocytometer. CRITICAL: Be careful not to overfill or create bubbles.
4. Count and record viable (clear) and dead cells (blue) of all four 16-square corners.

CRITICAL: If more than 200 cells/16 squares were counted, repeat count using a 1:5 or 1:10 dilution with 1X PBS or complete RPMI using a fresh sample aliquot.

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

D2 Protocol: Dead Cell Removal Using Ficoll

Materials Required

Complete RPMI (37°C)
Cells (Minimum 3x10^6)
2 x 15 mL Centrifuge Tubes
Lo-Bind Microcentrifuge Tube(s)
Ficoll Paque

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

1. Carefully add 6 mL of Ficoll to the bottom of the required number of 15 mL centrifuge tube(s) prior to harvesting stimulation cultures.
2. Centrifuge cells for 10 minutes at 300 rcf.
3. Remove cells from centrifuge, check for cell pellet.

- 4. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to aspirate remaining supernatant.
- 5. Resuspend the pellet(s) in 7 mL of complete RPMI. TIP: Be careful not to create bubbles.
- CRITICAL: Do not use more than 1 x 10^7 cells of your suspension per Ficoll tube.
- 6. Add the cell suspension(s) VERY SLOWLY to the tube(s) containing Ficoll. CRITICAL: Place the tip of your pipette on the wall of the tube, close to the Ficoll layer. Add cell suspension VERY SLOWLY.
- CRITICAL: This step must be done carefully and slowly to avoid mixing of the layers.

7. Centrifuge tubes for 20 minutes at 300 rcf without brake or acceleration.

- CRITICAL: Turn acceleration and brakes off to preserve the density layers established during centrifugation.

8. While cells centrifuge, prepare appropriate number of 15 mL centrifuge tube(s) containing 6 mL of complete RPMI.
9. Remove cells from centrifuge, check for cloudy layer which are the viable cells.

- 10. Aspirate a small volume of the supernatant. CRITICAL: Be careful not to aspirate cloudy layer containing viable cells.

11. Using a P1000 pipette, collect the viable cells by recovering the cloudy layer between Ficoll and complete RPMI media
12. Transfer cells into the 15 mL centrifuge tube(s) containing complete RPMI.

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

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 • Poor cell removal from plate • Trypan Blue is toxic	• Use recommended cell concentrations during incubation (**Chapter 7**) • 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. • Thoroughly mix cells in well with pipette prior to transferring to tube (refer to **step 9.2**) • 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	• 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 **Chapters 6 and 9** • Use only freshly prepared cell stain 405 per **Chapter 6**

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 **10.2** to avoid introduction of bubbles on chip
		• Ensure use of a sterile space to reduce introduction of potential contaminants. Use dedicated pipettes, tips, and tubes for sterile work. Pipette up and down gently and throughout protocol to reduce clumps.
		• Follow closely the Critical steps and tips in **Chapter 5** (Pan T Sample Enrichment)
		• Load recommended number of cells (30,000 cells per chip) (**Chapter 10**)
		• 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 anti-CD3/anti-CD28 or Cell Activation Cocktail stimulation concentration was not used • Recommended anti-CD3/anti-CD28 or Cell Activation Cocktail stimulation duration was not used	• Use anti-CD3/anti-CD28 or Cell Activation Cocktail concentrations listed in Chapter 2 and Chapter 7 • Use anti-CD3/anti-CD28 or Cell Activation Cocktail timing listed in Chapter 7 • Use recommended vendor as listed in Table 3