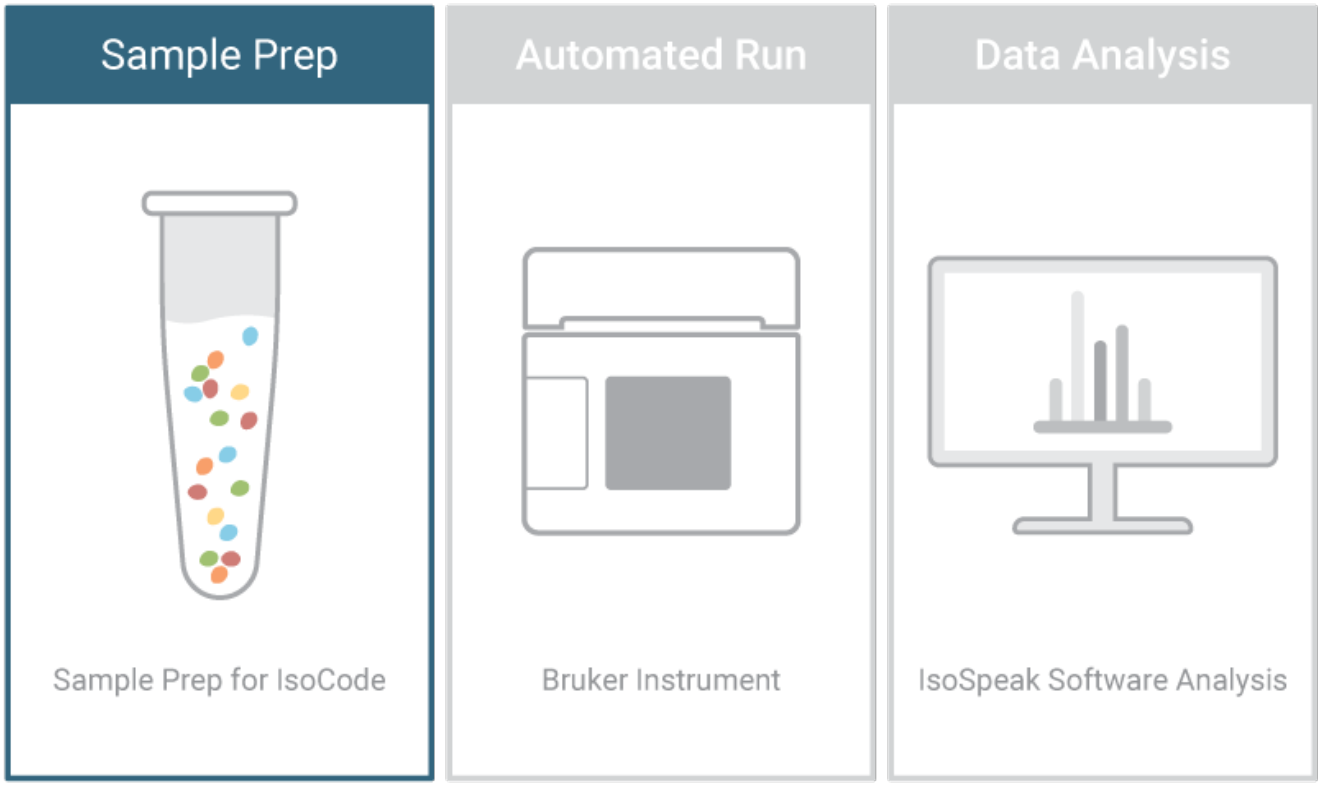
IsoCode Single-Cell Adaptive Immune: Human CD154+ T Cell Protocol

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

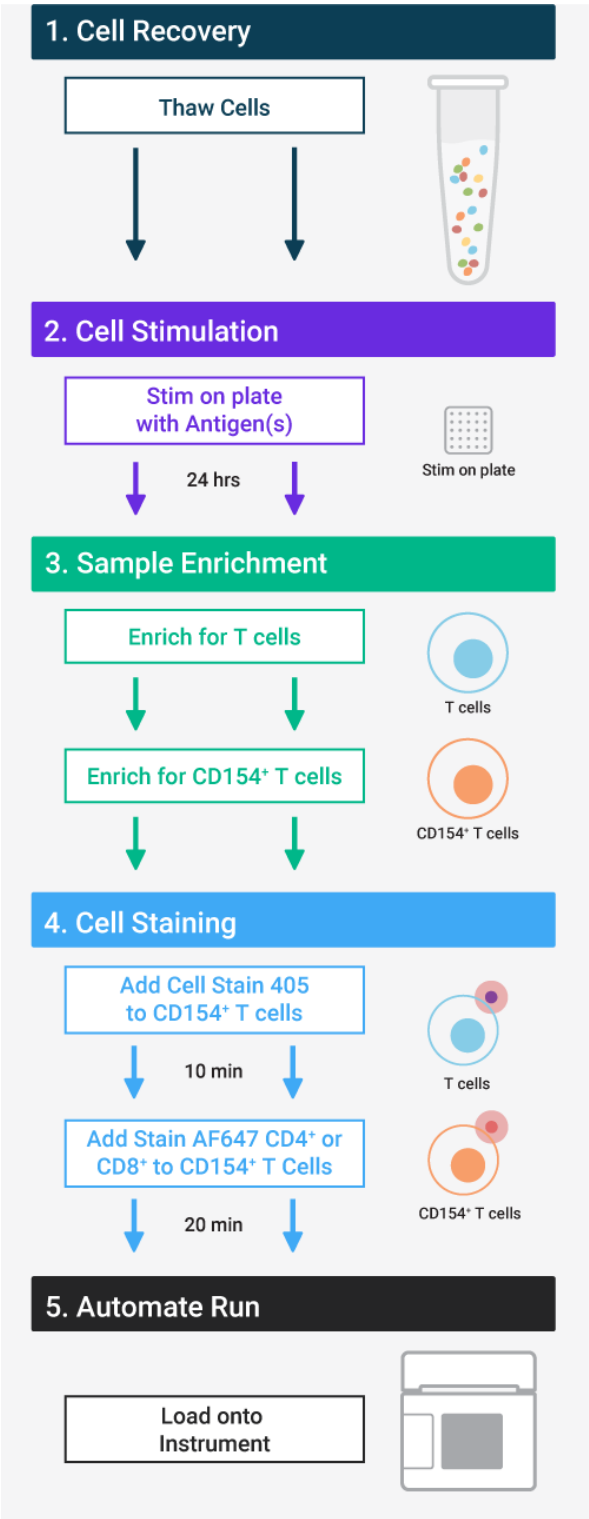
Overview of Protocol

Day 1: Cryopreserved cells are thawed and immediately Stimulated for 24 hours.

Day 2: Enrichment, Staining, and Loading of CD154⁺ T cells onto IsoCode chip.

NOTE:
This protocol outlines the standard method for thawing and culturing of human CD154⁺ T cells only and may not be valid for other species or cell types.

NOTE:
Using stains and protocols other than recommended stain and protocols might result in failed runs. Stains and staining procedures not approved by Bruker will require validation prior to use. Please consider Bruker's IsoPACE program to assist in custom marker and protocol validation.



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PRO-24 REV 6.0

Safety Warnings

- Read MSDS documents of all materials prior to use.

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

Required Reagents, Consumables and Equipment

Table 1: Required Reagents and Consumables Provided by Bruker

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

IsoCode Kit Components

IsoLight IsoCode Reagent Box (4°C)

15 mL Tube A

15 mL Tube B

1.5 mL Tubes A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)

50 mL Tubes containing Reagents 1, 2, 3, 4, 5, 6, 7, 8

1 Bag of Disposable Reagent Sippers

Alexa Fluor 647 anti-human CD4 stain (AF647-CD4) [ordered separately]

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 CD4 stain (AF647-CD4) [ordered separately]

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
96 Well Plate U-Bottom	N/A	Corning	353077
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
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 with GlutaMAX and HEPES	1x	ThermoFisher	72400047
Penicillin-Streptomycin Solution	100x	ThermoFisher	15140122
HyClone FBS	1x	Cytiva	SH30070.03IH30-45
Sodium Pyruvate	100x	ThermoFisher	11360070
MEM Vitamin Solution	100x	ThermoFisher	11120052
MEM Non-Essential Amino Acids Solution	100x	ThermoFisher	11140050
2-Mercaptoethanol	55 mM	ThermoFisher	21985023
Gentamicin	50 mg/mL	ThermoFisher	15750060
Recombinant Human IL-2	N/A	Peprotech	200-02
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
Antigen(s)		Experiment dependent	
Miltenyi Pan T Cell Isolation Kit, Human	N/A	Miltenyi	130-096-535
Miltenyi CD154 Microbead Kit, Human	N/A	Miltenyi	130-092-58
FcR Blocking Reagent, Human	N/A	Miltenyi	130-059-901
Accutase	1x	Innovative Cell Technologies, Inc	AT 104-500
Trypan Blue	0.4%	Gibco	15250-061
Reagent Alcohol 70%	N/A	Lab Grade	N/A

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

Table 4: Cell Staining Reagents

Test Material	Catalog Number	Color
Cell Stain 405	STAIN-1001-1	Violet
Alexa Fluor 647 anti-human CD4 (**AF647-CD4**)	STAIN-1002-1	Red
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

Key: ● TIP, ● CRITICAL, ● OPTIONAL

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. Reagent to Be Prepared Before Starting

Table 7: Complete CD154 RPMI Recipe

- CRITICAL: Complete CD154 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
RPMI with GlutaMAX and HEPES	1x	1x	429.45 mL	ThermoFisher/72400047
HyClone FBS	100%	10%	50 mL	Cytiva/SH30070.03IH30-45
Penicillin-Streptomycin Solution	100x	1x	5 mL	ThermoFisher/15140122
Sodium Pyruvate	100x	1x	5 mL	ThermoFisher/11360070
MEM Vitamin Solution	100x	1x	5 mL	ThermoFisher/11120052
MEM Non-Essential Amino Acids Solution	100x	1x	5 mL	ThermoFisher/11140050
2-Mercaptoethanol	55 mM	0.05 mM	454.5 µL	ThermoFisher/21985023
Gentamicin	50 mg/mL	10 µg/mL	100 µL	ThermoFisher/15750060
Recombinant Human IL-2	N/A	30 IU/mL	6.88 µg*	Peprotech/200-02

*The stated amount is based on an activity of 2.18 IU/ng. Please note that the IU/ng conversion may vary by lot. Check with vendor for applicable details.

Note | Add all components except IL-2. Add IL-2 powder last and stir solution until IL-2 powder is dissolved.

Sterile-filter complete CD154 RPMI media through 0.20 µm filter before use.

Store complete CD154 RPMI media at 4°C and warm up to 37°C in water bath prior to use.

Additional Reagents to Be Prepared

NOTE: 1X Accutase should be thawed overnight at 4°C upon receipt. 12 mL aliquots are recommended and storage is at -20°C. Thawed aliquots can be stored at 4°C for up to 1 month.

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 CD4 stain (AF647-CD4) [ordered separately]
- 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 CD4 stain (AF647-CD4) [ordered separately]
- 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: Recovery of Cryopreserved Cells

Materials Required

Complete CD154 RPMI (37°C)
Cryopreserved PBMC
15 mL Centrifuge Tube

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 CD154 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 CD154 RPMI in 15 mL centrifuge tube, labeled *Thawed PBMC*. TIP: Insert tip into complete CD154 RPMI when pipetting, be careful to not create bubbles.
- 6. Take 1 mL of complete CD154 RPMI and pipette into original thawed cell vial. Rinse inside the vial with the complete CD154 RPMI to recover additional thawed cells. TIP: Insert tip into complete CD154 RPMI, be careful to not create bubbles.
- 7. Draw up cell/complete CD154 RPMI mixture and pipette into the 15 mL centrifuge tube, labeled *Thawed PBMC*. TIP: Insert tip into complete CD154 RPMI and pipette gently up and down. Be careful to not create bubbles.
8. Centrifuge cells for 10 minutes at 300 rcf.
9. After cells are centrifuged, check for cell pellet.
- 10. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to remove last bit of supernatant.
11. Resuspend cell pellet in 0.5 mL of fresh complete CD154 RPMI.
- a. Mix well to resuspend. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
12. Slowly add additional complete CD154 RPMI to a final concentration of 6 x 10^6 cells/mL.
- 13. Mix with serological pipette. TIP: Gently pipet up and down 3-5 times, be careful to not create bubbles.
14. Proceed immediately to next chapter.

Chapter 3: Cell Stimulation

Materials Required

96 Well Plate U-bottom
1X Accutase (-20°C)
Antigen(s)
Thawed PBMCs from Chapter 2

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

Methods

1. Add 180 µL of cell suspension to desired number of wells on plate. Replicates should be plated if cell number allows.
2. Add 20 µL of antigen(s) to each of the wells containing cell suspension for stimulation on plate. CRITICAL: Be careful not to create bubbles. This will maximize even stimulation of cell suspension.
3. Use a pipette to mix the antigen(s) and cell suspension to ensure it is evenly distributed. TIP: Be careful not to create bubbles.
4. Add 200 µL of cell suspension to desired number of wells for control condition on plate if required for your specific experiment. Replicates should be plated if cell number allows. Note: No antigen(s) are added to these wells.
5. Incubate plate for 24 hours at 37°C, 5% CO_2.
6. Place 1X Accutase at 4°C to allow 1X Accutase to thaw overnight.

Chapter 4: Post-Stimulation Sample Setup

Materials Required

Complete CD154 RPMI (37°C)
Sterile 1X PBS (Room Temperature)
1X Accutase (Room Temperature)
15 mL Centrifuge Tube
Plate Containing Stimulated & Control PBMC from Chapter 3
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. Remove 1X Accutase from 4°C to allow 1X Accutase to come to room temperature. NOTE: 1X Accutase aliquots, after thaw, can be stored at 4°C for up to 1 month. Do not use 1X Accutase that has been at 4°C for longer than a month.
2. Remove plate from incubator.
3. Pipette non-adherent PBMC into a 15 mL centrifuge tube. NOTE: Pool wells if there are replicates into one tube.
4. Centrifuge cells for 10 minutes at 300 rcf.
5. While cells are centrifuging, add 100 µL of 1X PBS to each well to gently rinse adherent cells.
6. Remove the centrifuged cells and check for cell pellet.

OPTIONAL: Collect supernatants (100 µL) from all groups and store at -80°C for bulk assay.

7. Transfer 1X PBS from each well to 15 mL centrifuge tube. TIP: Aspirate from the edge of the well

Key: ● TIP, ● CRITICAL, ● OPTIONAL

8. Pipet 1X Accutase up and down 5 times to ensure it is well mixed.
9. Detach adherent cells from the plate by adding 50 µL of 1X Accutase to each well.
10. Rock the plate back and forth to ensure the 1X Accutase covers the entire bottom of the well.
11. Incubate the cells with 1X Accutase for 10 minutes at room temperature to allow the cells to detach. During incubation, rock the plate back and forth about once per minute.
12. After incubation, check under microscope to determine if cells are detached. If so, proceed to next step, if not incubate at room temperature and continue to check every 1 to 2 minutes.
13. Rinse each well with 100 µL of complete CD154 RPMI.
14. Transfer cell suspension from each well to 15 mL centrifuge tube containing the nonadherent cells and supernatant from step 7.
15. Centrifuge cells for 10 minutes at 300 rcf.
16. Remove the centrifuged cells and check for cell pellet.
- 17. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
18. Resuspend cell pellet in 5 mL of complete CD154 RPMI.
- 19. Mix well by pipetting up and down 5 times. TIP: Be careful not to create bubbles.
- 20. 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.
- 21. 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.
- CRITICAL: If cells are less than 80% viable, proceed to Appendix D2 Dead Cell Depletion Protocol using Ficoll.
22. Proceed immediately to next chapter.

Chapter 5: Pan T Cell 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 at room temperature for 5 minutes.

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 at room temperature for 10 minutes.

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 Chapter 6 or Chapter 7.

Chapter 6: FcR Blocking (Optional)

Materials Required

RoboSep Buffer (4°C)
FcR Blocking Reagent, Human (4°C)
T Cell Fraction from Chapter 5

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 60 µL RoboSep (4°C) and 20 µL of FcR Blocking Reagent (4°C).

3. Add 60 µL of cold RoboSep to 15 mL centrifuge tube containing 1 x 10^7 or fewer cells.
4. Add 20 µL of Miltenyi FcR Blocking Reagent and mix well by gently pipetting up and down 5 times.
5. Incubate at room temperature for 1 minute.
6. Proceed immediately to Chapter 7, step 4.

Chapter 7: CD154+ T Cell Subset Enrichment

Materials Required

Complete CD154 RPMI (37°C)
RoboSep Buffer (4°C)
Miltenyi CD154 MicroBead Kit, Human:
    1 mL CD154-Biotin, Human (4°C)
    2 mL Anti-Biotin MicroBeads UltraPure
MACS LS Column
T Cell Fraction with FcR Blocking Reagent from Chapter 6 or T Cell Fraction from Chapter 5
Enrichment Kit:
    MACS Metal Plate/Magnet Kit
    3 x 15 mL Centrifuge Tubes (*Discard, Flow Through, CD154 fraction*)
    Lo-Bind Microcentrifuge Tube for Post-Enrichment CD154

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

Methods

- CRITICAL: If Chapter 6 was completed, proceed immediately to step 4.

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).

3. Add 40 µL of cold RoboSep to 15 mL centrifuge tube containing 1 x 10^7 or fewer cells.
- CRITICAL: For every 1 x 10^7 cells, add 10 µL of CD154 Biotin (4°C).

4. Add 10 µL of Miltenyi CD154 Biotin and mix well by gently pipetting up and down 5 times.
5. Incubate at room temperature for 15 minutes.
- 6. After 15 minutes, add 1 mL of cold RoboSep. TIP: Not necessary to mix for this step.

7. Centrifuge cells for 10 minutes at 300 rcf.
8. Remove the centrifuged cells and check for cell pellet.
9. 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 80 µL RoboSep (4°C) and 20 µL of Anti-Biotin MicroBeads UltraPure (4°C).

10. Add 80 µL of cold RoboSep to 15 mL centrifuge tube containing 1 x 10^7 or fewer cells.
11. Add 20 µL of Miltenyi Anti-Biotin MicroBeads UltraPure and mix well by gently pipetting up and down 5 times.

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

12. Incubate at room temperature for 15 minutes.
13. After 15 minutes, add 2 mL of cold RoboSep. TIP: Not necessary to mix for this step.
14. Centrifuge cells for 10 minutes at 300 rcf.

TIP: Keep RoboSep in refrigerator during enrichment process.

15. 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.
16. After cells are centrifuged, check for cell pellet and continue with MACS separation.
17. 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.
18. 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.

19. 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.
20. 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.
21. 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.
22. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.

23. Mix cell suspension by gently pipetting up and down 5 times. NOTE: This ensures that the cells are evenly dispersed after sitting.
24. Draw up all 500 µL of cell suspension and pipette carefully into the center of the LS column without touching sides of the column.
25. 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 24. 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.
26. 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 "CD154 fraction."
27. Cap the "Flow Through" tube and discard.
28. Add 5 mL of cold RoboSep to the LS column. CRITICAL: Be careful not to touch the sides.
29. Take plunger, smoothly push down on the plunger to push the RoboSep buffer through the LS Column. TIP: Lift up at the end of the plunging action so that the liquid does not splash back onto the LS Column tip.
30. Set LS column back on the "CD154 fraction" tube.
31. 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.
32. Add another 4 mL of cold RoboSep to the LS Column.
33. 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.
34. Discard LS Column and plunger.
35. Centrifuge "CD154 fraction" tube for 10 minutes at 300 rcf.
36. After cells are centrifuged, check for cell pellet.
37. Aspirate RoboSep buffer from "CD154 fraction" tube. TIP: Be careful to not aspirate cell pellet.
38. Use pipette to aspirate the remaining supernatant from each tube. TIP: Be careful to not aspirate cell pellet.
39. Add 1 mL complete CD154 RPMI to "CD154 fraction" and resuspend cell pellet. TIP: Make sure there are no clumps or bubbles.
40. Aliquot 10 µL of the CD154 fraction into a Lo-Bind Microcentrifuge tube and proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

41. Move “CD154 fraction” tube to incubator until Cell Staining (Chapter 9).

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: Cell Staining

NOTE: Please read before proceeding with cell staining.

There are 2 steps for cell staining:

1. Cell staining (violet) as described in Chapter 9a.
2. CD8 or CD4 specific surface marker staining as described in Chapter 9b.

Cell Staining Reagents

Test Material	Catalog Number	Color
Cell stain 405	STAIN-1001-1	Violet
Alexa Fluor 647 anti-human CD4 (AF647-CD4)	STAIN-1002-1	Red
Alexa Fluor 647 anti-human CD8 (AF647-CD8)	STAIN-1003-1	Red

Chapter 9a: Cell Staining (Violet)

Materials Required

CD154 T Cells from Chapter 7
Lo-Bind Microcentrifuge Tubes (*Stain Master Mix, CD154*)
Sterile 1X PBS (Room Temperature)
Complete CD154 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 CD154 T cells from incubator.
4. Mix CD154 T cells by pipetting up and down. Transfer cells to a Lo-Bind microcentrifuge tube.
5. Centrifuge cells for 10 minutes at 300 rcf.
6. After cells are centrifuged, check for cell pellets.
- 7. Remove the majority of the supernatant with a P1000 or P200 pipette. Switch to a P10 pipette to remove all media from the cell pellet. TIP: Be careful not to aspirate the cell pellets.
8. Add 1 mL of PBS to each tube to dilute any remaining media and mix by pipetting up and down.

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

9. Centrifuge cells for 10 minutes at 300 rcf.
10. After cells are centrifuged, check for cell pellets.

- 11. Remove the majority of the supernatant with a P1000 or P200 pipette. Switch to a P10 pipette to remove all media from the cell pellet. TIP: Be careful not to aspirate the cell pellets.
12. Gently remix stain master mix.
- CRITICAL: Failure to remix stain master mix will result in poor staining.
- 13. For every 1 x 10^6 cells, add 100 µL of well mixed stain master mix to each cell suspension tube. CRITICAL: Pipet up and down 15 times to mix the cells. Be careful to not create bubbles.
14. Incubate for 5 minutes at 37°C in the dark.
- 15. Gently pipet to mix the cell suspension 15 times. CRITICAL: Be careful to not create bubbles.
16. Incubate for an additional 5 minutes at 37°C in the dark.
- 17. After incubation, add 5 times the volume of complete CD154 RPMI. CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.
18. Incubate for 10 minutes at 37°C in the dark.
- 19. Take 10 µL of cells to count. Count cells using a hemocytometer and determine percent of viable cells as described in Appendix D1. TIP: Cell counting can be done while cells are incubating.
20. Centrifuge stained cells for 10 minutes at 300 rcf.
21. After cells are centrifuged, check for cell pellets.
- 22. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
23. Proceed immediately to Chapter 9b.

Chapter 9b: Surface Marker-Specific Staining

Materials Required

Cells Stained with Cell Stain 405 from Chapter 9a
Complete CD154 RPMI (37°C)
AF647 anti-human CD4 or AF647 anti-human CD8 (4°C)
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

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

1. Resuspend cell pellets with 18 µL complete CD154 RPMI for 1 x 10^5 or fewer cells.
2. Spin tube of AF647 anti-human CD4 or CD8 in a mini centrifuge for 10 seconds to collect stain at the bottom of the tube. NOTE: Only one stain can be selected for use and stain is specific to desired cell subset for staining.

- 3. Add 2 µL of AF647 anti-human CD4 or CD8 to CD154+ samples (1:10 final dilution) for 1 x 10^5 or fewer cells. Mix gently by pipetting up and down. CRITICAL: Be sure to use appropriate stain for desired cell subset.
4. Incubate for 20 minutes at room temperature in the dark.
- 5. After incubation, add 1 mL of complete CD154 RPMI to each sample tube. TIP: Mix gently, be careful not to create bubbles.
- 6. 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.
7. Centrifuge stained cells for 10 minutes at 300 rcf.
8. After cells are centrifuged, check for cell pellets.
- 9. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
10. Resuspend the cells with complete CD154 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
Stained CD154 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 CD154 fractions by gently pipetting up and down 15 times. Immediately proceed to chip loading. 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. 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 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 EXCEPT prior to loading cells on chip due to spectral overlap of the stains. Manual cell counting is required prior to loading on the chip.

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

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

CRITICAL: If more than 200 cells/16 squares were counted, repeat count using a 1:5 or 1:10 dilution with 1X PBS or complete CD154 RPMI using a fresh sample aliquot.

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

Key: ● TIP, ● CRITICAL, ● OPTIONAL

D2 Protocol: Dead Cell Removal Using Ficoll

Materials Required

Complete CD154 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 CD154 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 CD154 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 CD154 RPMI media.
12. Transfer cells into the 15 mL centrifuge tube(s) containing complete CD154 RPMI.

- 13. Aliquot 10 µL of cell/complete CD154 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 • Trypan Blue is toxic • Poor cell removal from plate	• Use recommended cell concentrations during cell thawing (**Chapter 2**) • 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 • Follow cell removal steps as highlighted in **Chapter 4**
Low quality cell count on chip *Stain Process related*	• Use of media other than the recommended media in protocol which could interact with cell stain • Use of stains not recommended in protocol • Recommended stain concentration, incubation time and/or incubation temperature not used • Cell stain 405 was stored prior to use • Media not completely removed from cell pellet prior to staining	• Use complete CD154 RPMI media following recipe in **Table 7** • Use Bruker provided validated stain (**Table 4: Cell Staining Reagents**) • Follow staining steps as highlighted in **Chapter 9** • Use only freshly prepared cell stain 405 per **Chapter 9** • Ensure all media is removed from cell pellet in **step 9a.7**

Low quality cell count on chip *Technique Detail related*	• Bubbles loaded onto chip, especially at Chip Loading
	• Detection of potential artifacts such as debris, cell clumping, inefficient enrichment possibly due to:
	  ○ Pipetting wrong concentration
	  ○ Not fully inserting column into MACS separator
	  ○ Reagents not stored at recommended temperatures
	• Recommended number of cells not loaded on chip
	• Cell pellet or cells lost during centrifuging
		• Follow Critical step in **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 **Chapters 5 and 7** (Pan T Cell and CD154+ T Cell Enrichments)
		• Load recommended number of cells (30,000 cells per chip) (**Chapter 10**)
		• Use low protein binding centrifuge tubes

Key: ● TIP, ● CRITICAL, ● OPTIONAL

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 CD154 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])
Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Stimulation step related*	• Recommended antigen(s) stimulation duration was not used	• Use antigen(s) timing listed in Chapter 3

Key: ● TIP, ● CRITICAL, ● OPTIONAL