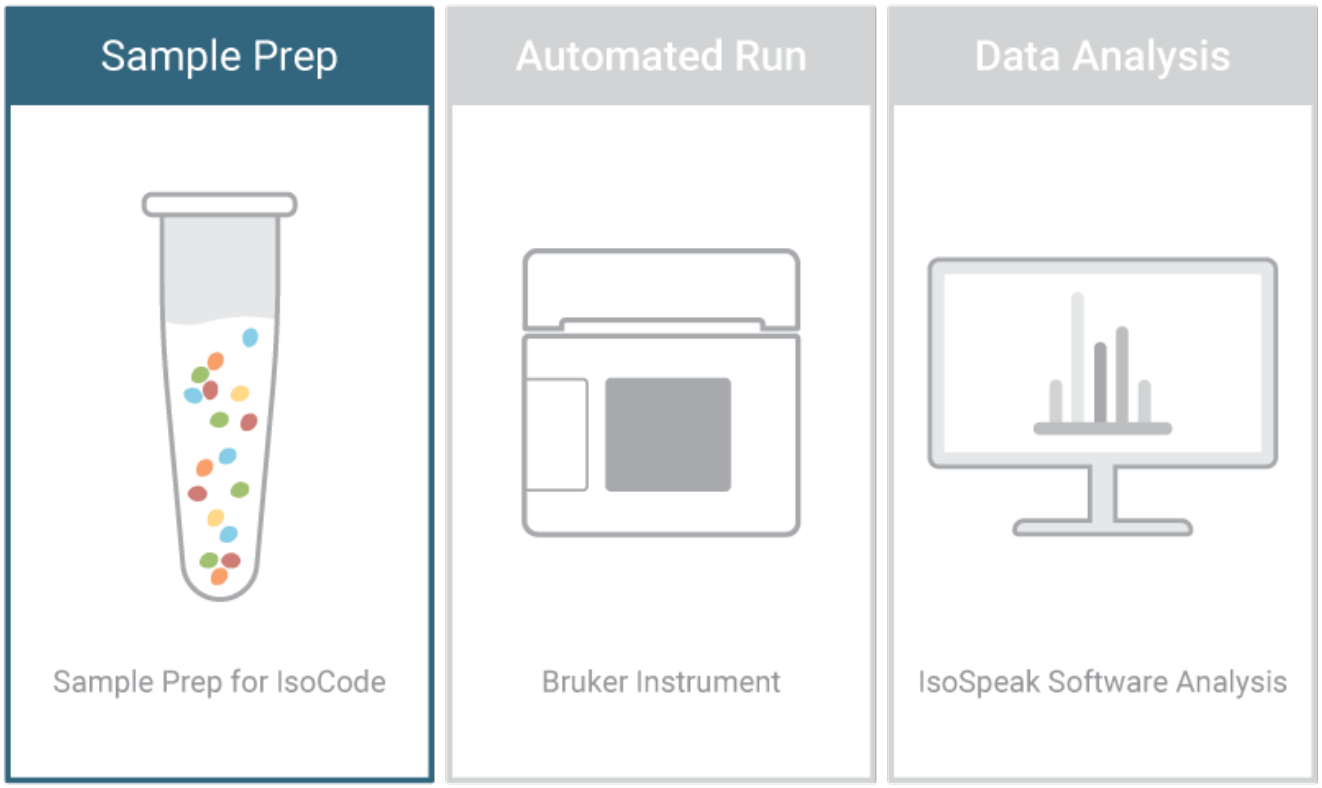
IsoCode Single-Cell Adaptive Immune: Mouse Bone Marrow (BM) CD117+ (cKIT) Cells Protocol

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

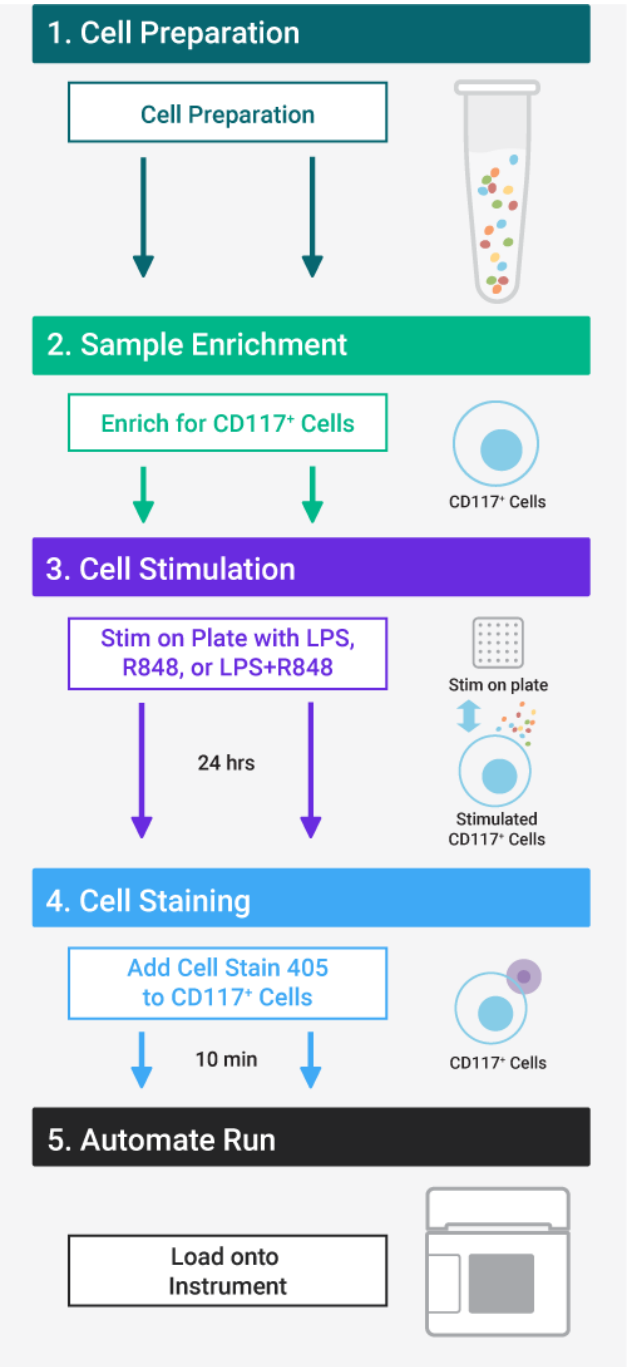
Overview of Protocol

Day 1: Mouse total BM cells are prepared. Enrichment and Stimulation of CD117+ (cKIT) cells for 24 hours.

Day 2: Staining and Loading of CD117+ cells onto IsoCode Chip.

NOTE:
This protocol outlines the standard method for thawing and culturing of mouse BM CD117+ 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.



Key: ● TIP, ● CRITICAL, ● OPTIONAL
PRO-28 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

IsoSpark IsoCode Reagent Box (4°C)

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

IsoCode Chip Set (-20°C)

- Boxes of IsoCode Chips (2 per box)
  - IsoSpark: 4 chip kits
  - IsoLight: 4 or 8 chip kits
- Cell Stain 405 [ordered separately]
- Cell Stain 405 Diluent (DMSO) [part of cell stain 405 kit]

Table 2: Required Consumables Not Supplied by Bruker

Consumable	Type	Source	Catalog Number
6 Well Plate Flat Bottom	N/A	Corning	353046
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
Centrifuge Tubes*	Conical, 50 mL	VWR	10026-078
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
Cell Strainer	70 µm	Fisher Scientific	08-771-2
Syringe with Needles	3 mL Syringe, 22-Gauge Needle	Fisher Scientific	14-817-130
Scissors	6 inches	Fisher Scientific	08-951-20
Tweezers	4.5 inches	Fisher Scientific	12-000-157

*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

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Reagent	Stock Concentration	Source	Catalog Number
RPMI	1x	ThermoFisher	MT 10040CV
Penicillin-Streptomycin-Neomycin Solution Stabilized	100x	Sigma	P4083-100 mL
FBS	1x	Sigma	F2442-6X500 mL
2-Mercaptoethanol	55 mM	Gibco	21985-023 50mL
Glutamax	100x	Thermo	35050061
LPS (lyophilized powder)	N/A	Sigma	L2654-1MG
R848 (Resiquimod, lyophilized)	N/A	InvivoGen	tlrl-r848-5
Endotoxin-free Water	1x	InvivoGen	tlrl-r848
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	Gibco	10010072
RoboSep Buffer	1x	StemCell Tech	20104
Ficoll Paque PREMIUM 1.084	N/A	Cytiva	17544602
Miltenyi CD117 Microbeads, Mouse	N/A	Miltenyi	130-091-224
ACK Lysis Buffer	1x	ThermoFisher	A10492-01
Trypan Blue	0.4%	Gibco	15250-061
Reagent Alcohol 70%	N/A	Lab Grade	N/A

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

Table 4: Cell Staining Reagents

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

Table 5: Required Equipment

Key: ● TIP, ● CRITICAL, ● OPTIONAL

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 except for during Chapter 1. In Chapter 1, centrifuges should be set to 4°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. Reagent 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

- CRITICAL: 2-Mercaptoethanol will be added to media used for all steps except cell staining quench media and final cell suspension volume to be loaded on IsoCode chip. 2-Mercaptoethanol should only be added to media right before use.

Note | Sterile-filter before use and store complete RPMI Media at 4°C and warm up to 37°C in water bath prior to use.

Table 8: LPS Recipe

CRITICAL: LPS has been validated for use by Bruker. Using alternative stimulant may result in failed runs. Please contact your Field Application Scientist for additional information.

Ingredient	Stock Concentration	Final Concentration	Amount for 1 mL	Vendor/Catalog
LPS	N/A	1 mg/ml	1 mg	Sigma/L2654-1MG
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	1x	1 mL	Gibco/10010072

- CRITICAL: Prepare 10 µL LPS aliquots and freeze at -20°C for no longer than 2 months. Aliquots are single use only and are to be thawed immediately prior to their usage. If there is any remaining volume in an aliquot, do not refreeze but discard.

Table 9: Working Stock of R848 (1 mg/mL) Recipe

Ingredient	Stock Concentration	Final Concentration	Amount for 500 µL*	Vendor/Catalog
Endotoxin-free Water	1x	1x	500 µL	InvivoGen/tlrl-r848
R848	N/A	1 mg/mL	500 µg	InvivoGen/tlrl-r848

*Vortex solution until R848 is completely dissolved.

- CRITICAL: Prepare 15 µL R848 aliquots and freeze at -20°C for no longer than 6 months. Aliquots are single use only and are to be thawed immediately prior to their usage. If there is any remaining volume in an aliquot, do not refreeze but discard.

Table 10: 2% FBS/PBS Recipe

Ingredient	Stock Concentration	Final Concentration	Amount for 1 L	Vendor/Catalog
FBS	100%	2%	20 mL	Sigma/F2442-6X500mL
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	1x	980 mL	Gibco/10010072

Note | Solution should be prepared same day and ideally right before starting the protocol. Sterile-filter before use and store at 4°C.

C. Protocol

Chapter 1: Getting Started

Kit Contents

IsoLight IsoCode Reagent Box (4°C)

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

IsoSpark IsoCode Reagent Box (4°C)

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

IsoCode Chip Set (-20°C)

- Boxes of IsoCode Chips (2 Per Box)
  - IsoSpark: 4 chip kits
  - IsoLight: 4 or 8 chip kits
- Cell Stain 405 [ordered separately]
- Cell Stain 405 Diluent (DMSO) [part of cell stain 405 kit]

Chapter 2: Mouse Total Bone Marrow Cells Preparation

Materials Required

Fresh Mouse Femurs
Sterile 2% FBS/PBS (Cold, on Ice)
Complete RPMI (37°C)
ACK Lysis Buffer (Room Temperature)
3-mL Syringe with 22-Gauge Needle
Scissors
Tweezers
70 µm Cell Strainer
15 mL Centrifuge Tube
50 mL Conical tube
6 Well Plate Flat Bottom

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 4°C. Ensure centrifuges are pre-cooled for the following steps.

Note: Mouse femurs should be processed as soon as possible upon harvest or receipt. All bones are processed in parallel. It is recommended to process a maximum of 10 bones per person at once.

Methods

1. Cut both ends of each bone and place each bone in a well of a 6 well plate containing 2-3 mL of 2% FBS/PBS.
2. With a 22G needle on a 3 mL syringe, draw up 3 mL of fresh 2% FBS/PBS.
3. Using tweezers, pick up one bone while holding the syringe in the other hand.
4. Insert the needle into the distal end of the bone. TIP: The distal end of the bone is the thinner end. Bone should appear red through the center.
5. Slowly and gently expunge the buffer through the bone and into a 50 mL conical tube. TIP: A good flush of the bone will yield a red plug into the conical tube.
6. Inspect the bone; it should now appear white. If red is still observed, complete an additional flush by repeating steps 2 through 5.
7. With a P1000, gently pipet the bone marrow mixture up and down to obtain a single-cell suspension.
8. Centrifuge cells for 5 minutes at 450 rcf at 4°C. NOTE: Centrifuge should be pre-cooled.
9. Remove the centrifuged cells and check for cell pellet.
10. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
11. Resuspend cell pellet in 1 mL ACK Lysis Buffer.
12. Incubate for 3 minutes at room temperature. Do not exceed 5 minutes.
13. Add 20 mL of 2% FBS/PBS.
14. Centrifuge cells for 5 minutes at 450 rcf at 4°C.
15. Remove the centrifuged cells and check for cell pellet.
16. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
17. Resuspend cell pellet in 5 mL of 2% FBS/PBS.
18. Place a 70 µm cell strainer on top of a new 15 mL centrifuge tube.
19. Pass the cell suspension through the strainer by slowly dripping the cell suspension from a pipette onto the strainer drop-by-drop.
20. Rinse the inside of the 50 mL conical tube and strainer with 5 mL of 2% FBS/PBS to recover additional cells.
21. Mix well 5 times with 10 mL serological pipette. TIP: Be careful not to create bubbles.
22. 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.
23. 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.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

- CRITICAL: If cells are less than 80% viable, proceed to Appendix D2 Protocol: Dead Cell Removal Using Ficoll.

NOTE: It is expected to recover 3-4 x 10^7 total bone marrow cells per pair of mouse femurs.

24. Remove the centrifuged cells and check for cell pellet.
- 25. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
26. Resuspend cell pellet in 5 mL 2% FBS/PBS.
27. Transfer cell suspension from the 50 mL conical tube to a 15 mL centrifuge tube.
28. Centrifuge cells for 10 minutes at 300 rcf.
29. Proceed immediately to next chapter.

Chapter 3: Sample Enrichment

Materials Required

Complete RPMI (37°C)
2-Mercaptoethanol (4°C)
RoboSep Buffer (4°C)
Miltenyi CD117 Microbeads, Mouse, 2 mL (4°C)
MACS LS Column
Prepared Cells from Chapter 2
Enrichment Kit:
    MACS Metal Plate/Magnet Kit
    3 x 15 mL Centrifuge Tubes (*Discard, Flow Through, CD117 fraction*)
    Lo-Bind Microcentrifuge Tube for Post-Enrichment CD117 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 80 µL RoboSep (4°C) and 20 µL of CD117 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 the Miltenyi CD117 Microbeads at a slow speed for 10 seconds.
5. Add 20 µL of Miltenyi CD117 Microbeads to the cell suspension in RoboSep (containing 1 X 10^7 or fewer cells) 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 2ml 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.
12. For 1 x 10^8 cells or fewer, 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

13. 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.
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 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.
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 of the column.
19. Wash LS 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 18. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

20. After the last drop of the wash passes through or does not fall, remove the LS Column carefully from the magnet, and place carefully on the tube labeled for "CD117 fraction."

● 21. Add 5 mL of cold RoboSep to the LS column. CRITICAL: Be careful not to touch the sides.

● 22. 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.

23. Set LS Column back on the "CD117 fraction" tube.

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

24. Loosen up plunger. Remove plunger briefly from column and hold in one hand.

25. Add another 2 mL of cold RoboSep to the LS Column.

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

27. Discard LS Column and plunger.

28. Centrifuge "CD117 fraction" tube for 10 minutes at 300 rcf.

29. Prepare complete RPMI supplemented with 55 µM of 2-Mercaptoethanol.

    a. Dilute 5 µL of 55 mM 2- Mercaptoethanol into 5 mL of complete RPMI.

    b. Use serological pipette to mix thoroughly.

● CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.

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

● 31. Aspirate RoboSep buffer from "CD117 fraction" tube. TIP: Be careful to not aspirate cell pellet.

32. Use pipette to aspirate the remaining supernatant. TIP: Be careful to not aspirate cell pellet.

● 33. Add 1 mL complete RPMI/2-Mercaptoethanol to "CD117 fraction" and resuspend cell pellet. TIP: Make sure there are no clumps or bubbles.

34. Add an additional 1 mL of complete RPMI/2-Mercaptoethanol and mix thoroughly by gently pipetting up and down 5 times. TIP: Make sure there are no clumps or bubbles.

● 35. Aliquot 10 µL of the "CD117 fraction" into a Lo-Bind Microcentrifuge tube and proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.

36. Move "CD117 fraction" tube to incubator until Cell Stimulation (Chapter 4).

Chapter 4: Cell Stimulation

NOTE: Please read before proceeding with cell stimulation.

There are 3 options for cell stimulation depending on your experimental design:

1. Cell stimulation with LPS as described in Chapter 4a
2. Cell stimulation with R848 as described in Chapter 4b.
3. Cell stimulation with LPS and R848 as described in Chapter 4c.

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

Chapter 4a: LPS stimulation

Materials Required

Complete RPMI (37°C)
2-Mercaptoethanol (4°C)
LPS 1 mg/ml (-20°C)
Sterile 1X PBS (Room Temperature)
15 mL Centrifuge Tube (*LPS CD117, Unstimulated CD117)*
Incubated CD117 Cells in Complete RPMI from Chapter 3
96 Well Plate Flat Bottom

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 supplemented with 55 µM of 2-Mercaptoethanol.
    a. Dilute 10 µL of 55 mM 2-Mercaptoethanol into 10 mL of complete RPMI.
    b. Use serological pipette to mix thoroughly.

● CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.

2. Thaw a vial of stock 1 mg/mL LPS at room temperature.

● 3. Vortex LPS stock at a slow speed for 10 seconds. TIP: Ensure contents are well-mixed.

● 4. Spin LPS stock in a mini centrifuge for 10 seconds. TIP: Ensure that contents are all in the bottom of the vial.

5. Prepare a working stock of LPS. Add 10 µL of 1 mg/mL LPS to 90 µL complete RPMI/2-Mercaptoethanol. Final concentration of LPS is 0.1 mg/mL. CRITICAL: Working 0.1 mg/mL LPS stock should be made fresh. If there is any remaining 1 mg/mL LPS stock, discard and do not re-freeze.

● 6. Vortex LPS working stock for 10 seconds. TIP: Ensure contents are well-mixed.

7. Remove CD117 cells from incubator.

8. Centrifuge CD117 cells for 10 minutes at 300 rcf.

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

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

Key: ● TIP, ● CRITICAL, ● OPTIONAL

11. Resuspend CD117 cells in complete RPMI/2-Mercaptoethanol to a cell density of 1 x 10^6 cells/mL.
12. Split cells into two separate 15 mL centrifuge tubes. One labeled "LPS CD117" and the other "Unstimulated CD117".
13. Mix cells in "Unstimulated CD117" tube by pipetting up and down gently 5 times. Plate 100 µl of the cell suspension per well in the 96 well plate. TIP: Plate at least two wells on the 96 Well Flat Bottom Plate for the unstimulated condition.
14. Take 10 µL from the "LPS CD117" tube and transfer into a Lo-Bind Microcentrifuge tube. Proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.
15. Centrifuge tube labeled "LPS CD117" for 10 minutes at 300 rcf.
16. After cells are centrifuged check for pellet.
17. Aspirate supernatant. TIP: Be careful to not aspirate cell pellet.
18. Resuspend the cell pellet with complete RPMI/2-Mercaptoethanol mixture supplemented with 100 ng/mL of LPS to a density of 1 x 10^6 cells/mL. CRITICAL: Volume is dependent on number of cells.
    a. Add 1 µL of 0.1 mg/mL LPS for every ml of RPMI/2-Mercaptoethanol. This yields a final concentration of LPS of 100 ng/ml.
    b. Use serological pipette to mix thoroughly. CRITICAL: Be careful not to create bubbles. This will maximize even stimulation of cell suspension.
19. Mix cells in "LPS CD117" tube by pipetting up and down gently 5 times. Add 100 µL of cell suspension to empty wells on the 96 well plate.
20. Incubate plate for 24 hours at 37°C, 5% CO_2.

Chapter 4b: R848 stimulation

Materials Required

Complete RPMI (37°C)
2-Mercaptoethanol (4°C)
R848 1 mg/ml (-20°C)
Sterile 1X PBS (Room Temperature)
15 mL Centrifuge Tube (*R848 CD117, Unstimulated CD117)*
Incubated CD117 Cells in Complete RPMI from Chapter 3
96 Well Plate Flat Bottom

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 supplemented with 55 µM of 2-Mercaptoethanol.
    a. Dilute 10 µL of 55 mM 2-Mercaptoethanol into 10 mL of complete RPMI.
    b. Use serological pipette to mix thoroughly.

- CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.

2. Thaw a vial of stock 1 mg/mL R848 at room temperature.
- 3. Vortex R848 stock at a slow speed for 10 seconds. TIP: Ensure contents are well-suspended.
- 4. Spin R848 stock in a mini centrifuge for 10 seconds. TIP: Ensure that contents are all in the bottom of the vial.
- 5. Prepare R848 and complete RPMI/2-Mercaptoethanol mixture by supplementing complete RPMI/2-Mercaptoethanol with 1 µg/mL of R848. CRITICAL: Volume is dependent on number of cells.
    a. Add 5 µL of 1 mg/mL R848 into 5 mL of complete RPMI/2-Mercaptoethanol. This yields a final concentration of R848 of 1 µg/mL.
    b. Use serological pipette to mix thoroughly.
6. Remove CD117 cells from incubator.
7. Centrifuge CD117 cells for 10 minutes at 300 rcf.
8. After cells are centrifuged, check for cell pellet.
- 9. Aspirate supernatant with pipette. TIP: Make sure to use a manual pipette to prevent accidental aspiration of cell pellet.
10. Resuspend CD117 cells in complete RPMI/2-Mercaptoethanol to a cell density of 1 x 10^6 cells/mL.
11. Split cells into two separate 15 mL centrifuge tubes. One labeled “R848 CD117” and the other “Unstimulated CD117”.
- 12. Mix cells in “Unstimulated CD117” tube by pipetting up and down gently 5 times. Plate 100 µl of the cell suspension per well in the 96 well plate. TIP: Plate at least two wells on the 96 Well Flat Bottom Plate for the unstimulated condition.
- 13. Take 10 µL from the “R848 CD117” tube and transfer into a Lo-Bind Microcentrifuge tube. Proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.
14. Centrifuge tube labeled “R848 CD117” for 10 minutes at 300 rcf.
15. After cells are centrifuged check for pellet.
- 16. Aspirate supernatant. TIP: Be careful to not aspirate cell pellet.
17. Use a pipette to mix the R848/complete RPMI/2-Mercaptoethanol mixture to ensure it is evenly distributed.
- 18. Using the R848/complete RPMI/2-Mercaptoethanol mixture from step 17, resuspend CD117 cells to a cell concentration of 1 x 10^6 cells/mL. TIP: Resuspend as thoroughly as possible, but gently.
19. Mix cells in “R848 CD117” tube by pipetting up and down gently 5 times. Add 100 µL of cell suspension to empty wells on the 96 well plate.
20. Incubate plate for 24 hours at 37°C, 5% CO_2.

Chapter 4c: LPS and R848 Stimulation

Materials Required

Complete RPMI (37°C)
2-Mercaptoethanol (4°C)
LPS 1 mg/ml (-20°C)
R848 1 mg/ml (-20°C)
Sterile 1X PBS (Room Temperature)
15 mL Centrifuge Tube (*LPS/R848 CD117, Unstimulated CD117)*
Incubated CD117 Cells in Complete RPMI from Chapter 3
96 Well Plate Flat Bottom

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 supplemented with 55 µM of 2-Mercaptoethanol.
    a. Dilute 10 µL of 55 mM 2-Mercaptoethanol into 10 mL of complete RPMI.
    b. Use serological pipette to mix thoroughly.

● CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.

2. Thaw vials of stock 1 mg/mL LPS and R848 at room temperature.

● 3. Vortex LPS and R848 stock at 1mg/ml at a slow speed for 10 seconds. TIP: Ensure contents are well suspended.

● 4. Spin LPS and R848 stock in a mini centrifuge for 10 seconds. TIP: Ensure that contents are all in the bottom of the vial.

5. Prepare a working stock of LPS. Add 10 µL of 1 mg/mL LPS to 90 µL complete RPMI/2-Mercaptoethanol. Final concentration of LPS is 0.1 mg/mL. CRITICAL: Working 0.1 mg/mL LPS stock should be made fresh. If there is any remaining 1 mg/mL LPS stock, discard and do not re-freeze.

6. Vortex LPS working stock for 10 seconds. TIP: Ensure contents are well-mixed.

7. Remove CD117 cells from incubator.

8. Centrifuge CD117 cells for 10 minutes at 300 rcf.

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

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

11. Resuspend CD117 cells in complete RPMI/2-Mercaptoethanol to a cell density of 1 x 10^6 cells/mL.

12. Split cells into two separate 15 mL centrifuge tubes. One labeled “LPS/R848 CD117” and the other “Unstimulated CD117”.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

- 13. Mix cells in "Unstimulated CD117" tube by pipetting up and down gently 5 times. Plate 100 µl of the cell suspension per well in the 96 well plate. TIP: Plate at least two wells on the 96 Well Flat Bottom Plate for the unstimulated condition.
- 14. Take 10 µL from the "LPS/R848 CD117" tube and transfer into a Lo-Bind Microcentrifuge tube. Proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.
15. Centrifuge tube labeled "LPS/R848 CD117" for 10 minutes at 300 rcf.
16. After cells are centrifuged check for pellet.
- 17. Aspirate supernatant. TIP: Be careful to not aspirate cell pellet.
- 18. Resuspend the cell pellet with complete RPMI/2-Mercaptoethanol mixture supplemented with 100 ng/ml LPS and 1 µg/ml R848 to a density of 1 x 10^6 cells/mL. CRITICAL: Volume is dependent on number of cells.
    a. Add 1 µL of 0.1 mg/mL LPS and 1 µL of 1 mg/ml R848 for every mL of RPMI/2-Mercaptoethanol.This yields a final concentration of 100 ng/ml of LPS and 1 µg/ml of R848.
    b. Use serological pipette to mix thoroughly.
19. Mix cells in "LPS/R848 CD117" tube by pipetting up and down gently 5 times. Add 100 µL of cell suspension to empty wells on the 96 well plate.
20. Incubate plate for 24 hours at 37°C, 5% CO_2.

Chapter 5: 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 7).
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 6: Cell Staining

Materials Required

Unstimulated and Stimulated CD117 Cells in 96 Well Plate from Chapter 4
Lo-Bind Microcentrifuge Tubes (*Stain Master Mix, CD117*)
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 96 well plate with CD117 cells from incubator.
4. Mix CD117 cells by pipetting up and down. Transfer cells to a Lo-Bind microcentrifuge tube by using P100 pipette to draw up 100 µL at a time in a gentle, circular motion until well is empty. NOTE: Pool wells if there are replicates.
5. Centrifuge cells for 10 minutes at 300 rcf.
6. After cells are centrifuged, check for cell pellets.

● 7. 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.

*NOTE: Supernatants may be stored at -80°C for bulk assay.

8. Add 1 mL of PBS to dilute any remaining media and mix by pipetting up and down.

● 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. Aspirate supernatant with a pipette. 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 to mix the cells 15 times. Be careful to not create bubbles.
14. Incubate for 5 minutes at 37°C in the dark.
- 15. Gently pipette 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 RPMI without 2-Mercaptoethanol. 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. Resuspend the cell pellet with complete RPMI without 2-Mercaptoethanol to a cell density of 1 x 10^6 cells/mL. Proceed to Chapter 7.

Chapter 7: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 5
Stained CD117 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 CD117 stimulated and unstimulated cell 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 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 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 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) 2- Mercaptoethanol (4°C) Cells (Minimum 3x10^6) 2 x 15 mL Centrifuge Tubes Lo-Bind Microcentrifuge Tubes Ficoll Paque PREMIUM 1.084

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 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. Prepare complete RPMI supplemented with 55 µM of 2-Mercaptoethanol.
    a. Dilute 6 µL of 55 mM 2-Mercaptoethanol into 6 mL of complete RPMI.
    b. Use serological pipette to mix thoroughly.
- CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.
9. While cells centrifuge, prepare appropriate number of 15 mL centrifuge tube(s) containing 6 mL of complete RPMI/2-Mercaptoethanol.
10. Remove cells from centrifuge, check for cloudy layer which are the viable cells.
- 11. Aspirate a small volume of the supernatant. CRITICAL: Be careful not to aspirate cloudy layer containing viable cells.
12. Using a P1000 pipette, collect the viable cells by recovering the cloudy layer between Ficoll and complete RPMI media
13. Transfer cells into the 15 mL centrifuge tube(s) containing complete RPMI.
- 14. 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.

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 appropriate dilutions recommended in Appendix D1. • Do a recount if initial count does not seem accurate • Quick spin Trypan Blue to pellet potential debris, remove aliquot from top of Trypan Blue. Start with fresh aliquot of Trypan Blue. • Count within 15 minutes of staining the cells • Follow cell removal steps as highlighted in Chapter 6
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 6 • Use only freshly prepared cell stain 405 per Chapter 6 • Ensure all media is removed from cell pellet in step 6.7

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
	  ○ Reagents not stored at recommended temperatures
	• Recommended number of cells not loaded on chip
	• Cell pellet or cells lost during centrifuging
		• Follow Critical steps in 7.2 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.
		• Load recommended number of cells (30,000 cells per chip) (Chapter 7)
		• 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 PREMIUM 1.084	• 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 2 to ensure protocol is being performed with the highest quality of cells. Use Ficoll Paque

	• Low viable cells due to lack of 2-Mercaptoethanol supplementation to complete RPMI. • Decreased viability due to cell shock • Recommended stimulation duration was not used	PREMIUM 1.084 in Appendix D2 if viability is less than 80% • Ensure that complete RPMI is supplemented with 2-Mercaptoethanol right before use. • Use reagents at recommended temperatures (i.e. always use warmed media [37°C]) • Use stimulation timing in Chapter 4