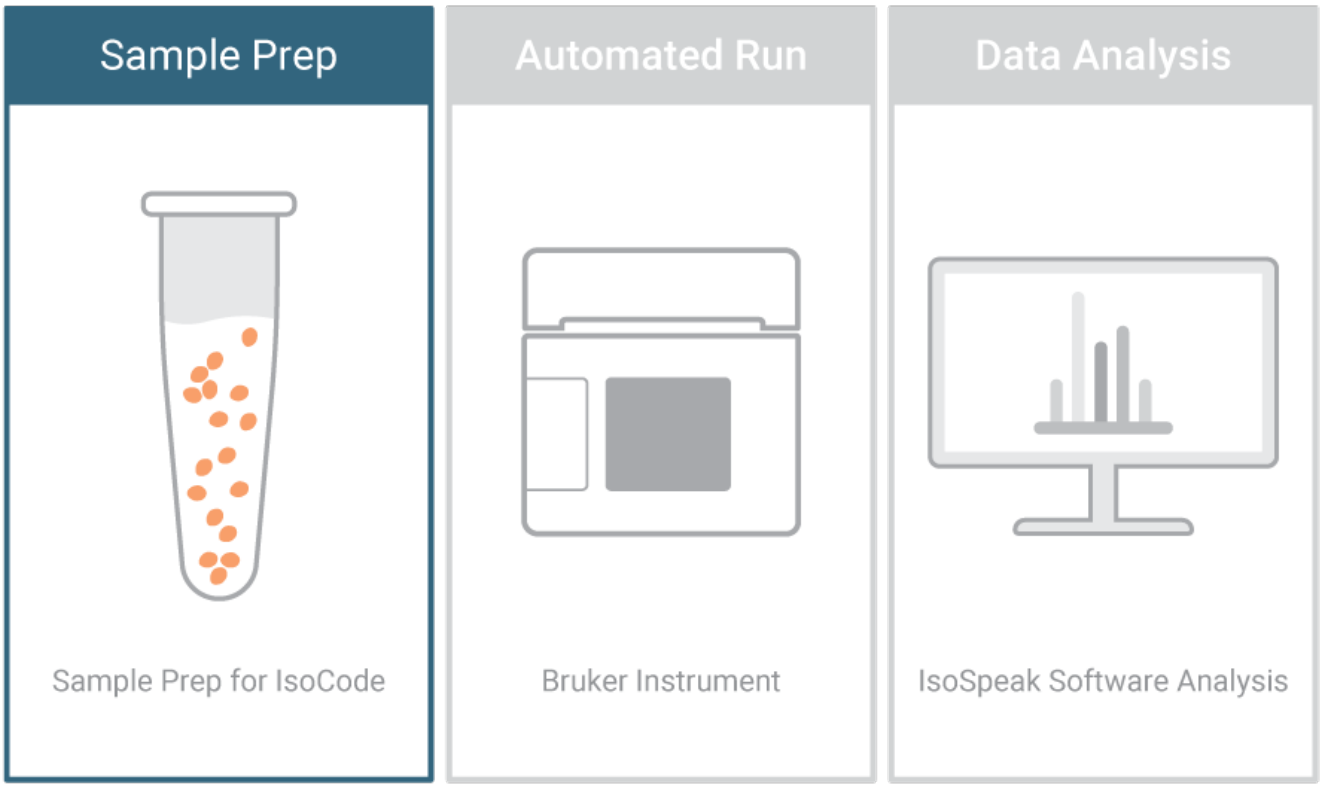
IsoCode Single-Cell Adaptive Immune: Human Tumor Associated Endothelial Cell (EC) Protocol

Ensure you achieve the maximum benefit from our systems and generate useful data ASAP that relates to in vivo immune activity



Key: ● TIP, ● CRITICAL, ● OPTIONAL

Contents

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A. Overview

Overview of Protocol

Day 1: Cryopreserved endothelial cells (ECs) are thawed and cultured overnight.

Day 2: ECs are seeded onto plate and cultured overnight.

Day 3: Stimulation of ECs for 24 hours.

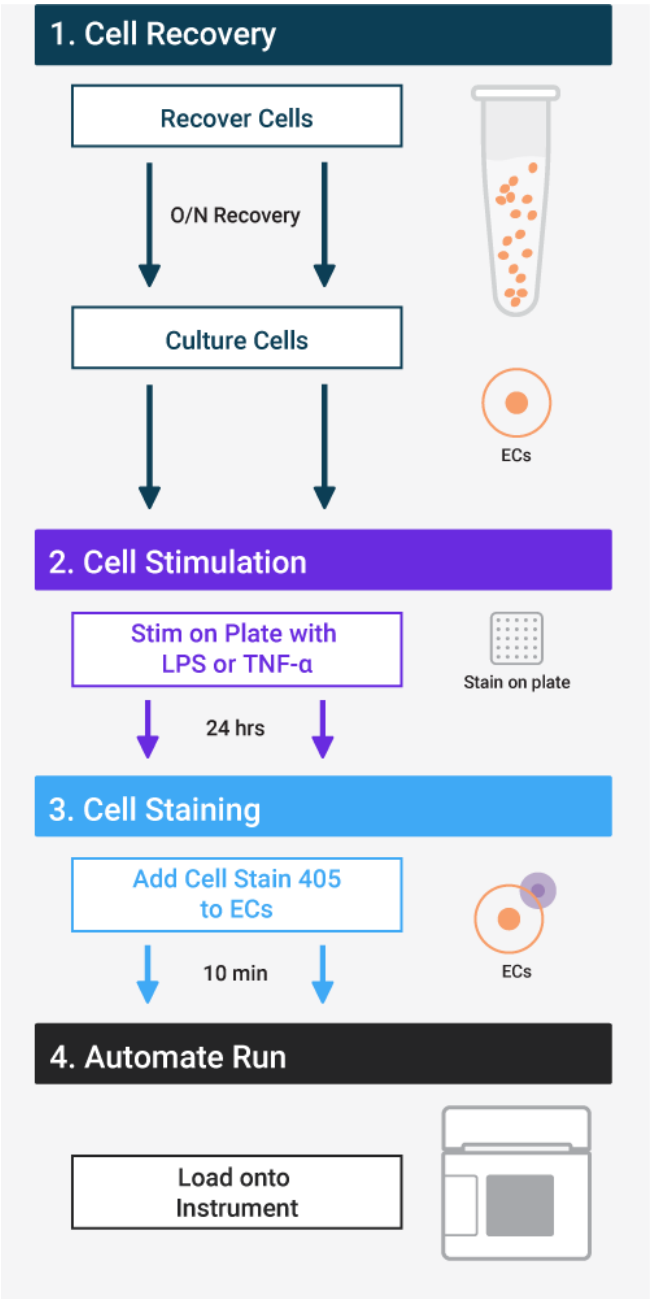
Day 4: Staining and Loading of ECs onto IsoCode Chip.

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

NOTE:
For brevity, when this protocol refers to endothelial cells (ECs), this is meant to refer exclusively to human tumor associated endothelial cells.

NOTE:
Cytokine profile may change over the course of multiple passages of primary cells. Stimulation time may vary and is dependent upon the experimental design and/or passage of the 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.



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
T25 Flask	N/A	Corning	29185-300
T75 Flask	N/A	Corning	430641U
24 Well Plate Flat Bottom	N/A	Corning	3524
6 Well Plate Flat Bottom	N/A	Corning	353046
12 Well Plate Flat Bottom	N/A	Corning	353043
48 Well Plate Flat Bottom	N/A	Corning	3548
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
EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 Bullet Kit	1x	Lonza	CC-3202
0.1% Gelatin in Water	1x	StemCell	07903
LPS (lyophilized powder)	N/A	Sigma	L2654-1MG
TNF-α (lyophilized powder)	N/A	Gibco	PHC3015
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	Gibco	10010072
Ficoll Paque Plus	N/A	GE Healthcare	17-1440-03
TrypLE Express Enzyme (1X), phenol red**	1x	ThermoFisher	12605010
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.

**Store TrypLE Express at room temperature and warm up to 37°C in water bath prior to use.

Table 4: Cell Staining Reagents

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

Table 5: Required Equipment

Equipment	Source	Catalog Number
IsoLight, IsoSpark, or IsoSpark Duo Instrument	Bruker	ISOLIGHT-1000-1, ISOSPARK-1000-1, or ISOSPARK-1001-1
Culture Hemocytometer	(Fisher) Hauser Levy	02-671-55A
Hemocytometer Cover Glass	(Fisher) Hauser Levy	02-671-53

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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: 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: TNF-α Recipe

- CRITICAL: TNF-α 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
TNF-α	N/A	0.1 mg/mL	0.1 mg	Gibco/PHC3015
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	1x	1 mL	Gibco/10010072

- CRITICAL: Prepare 10 µL TNF-α aliquots and freeze at -20°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.

Additional Reagents to Be Prepared

NOTE: Prepare EGM-2 MV media according to manufacturer's instructions prior to starting the protocol. Sterile-filter through 0.20 µm filter before use. Store complete EGM-2 MV Media at 4°C and warm up to 37°C in water bath prior to use.

C. Protocol

Chapter 1: Getting Started

Kit Contents

IsoLight IsoCode Reagent Box (4°C)

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

IsoSpark IsoCode Reagent Box (4°C)

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

IsoCode Chip Set (-20°C)

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

Chapter 2: Recovery of Cryopreserved Cells

Materials Required

0.1% Gelatin Solution (4°C)
EGM-2 MV Media (37°C)
Cryopreserved ECs
15 mL Centrifuge Tube
T25 Flask or T75 Flask

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

Methods

1. Remove 0.1% gelatin solution from refrigerator and allow solution to come to room temperature for 1 hour prior to use.
2. Coat flask with 0.1% gelatin solution and immediately remove solution. Leave flask to dry in the hood for 30 minutes.

- For T25 Flask add 5 mL
- For T75 Flask add 10 mL

3. Pipette 5 mL of EGM-2 MV media into a 15 mL centrifuge tube, labeled *Thawed ECs*.
4. Using proper PPE, remove cells from liquid nitrogen storage and thaw cells. TIP: Be careful of contamination.
5. 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.
6. 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.
7. Slowly pipette thawed cells into 5 mL of EGM-2 MV media in 15 mL centrifuge tube, labeled *Thawed ECs*. TIP: Insert tip into EGM-2 MV media when pipetting, be careful to not create bubbles.
8. Take 1 mL of EGM-2 MV media and pipette into original thawed cell vial. Rinse inside the vial with EGM-2 MV media to recover additional thawed cells. TIP: Insert tip into EGM-2 MV media, be careful to not create bubbles.
9. Draw up cell/EGM-2 MV mixture and pipette into the 15 mL centrifuge tube, labeled *Thawed ECs*. TIP: Insert tip into EGM-2 MV media and pipette gently up and down. Be careful to not create bubbles.
10. Centrifuge cells for 10 minutes at 300 rcf.
11. After cells are centrifuged, check for cell pellet.
12. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to remove last bit of supernatant.
13. Resuspend cell pellet in 0.5 mL of fresh EGM-2 MV media.
    a. Mix well to resuspend. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
14. Slowly add additional EGM-2 MV media to a final concentration of 1 x 10^5 cells/mL.
15. Transfer cell suspension to gelatin-coated flask. For 3-5 mL of cell volume, transfer to a T25 flask, or for 5-10 mL of cell volume, transfer to a T75 flask. TIP: Slowly pipette down the side of the flask as to not create bubbles.
16. Spread out cell suspension by rocking the flask carefully to fully cover the bottom. TIP: Be careful to not make bubbles.
17. Move to incubator at 37°C, 5% CO_2 until cells are at least 80% confluent.

Chapter 3: Cell Culture

Materials Required

0.1% Gelatin Solution (4°C)
Complete RPMI (37°C)
TrypLE Express (37°C)
Sterile PBS (Room Temperature)
Confluent Cells from Chapter 2 or Fresh ECs if Working with Fresh Samples
15 mL Centrifuge Tube
Lo-Bind Microcentrifuge Tube for Cell Count
48 Well, 24 Well, 12 Well or 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. Remove 0.1% gelatin solution from refrigerator and allow solution to come to room temperature for 1 hour prior to use.
2. Coat the desired plate with appropriate volume per well of 0.1% gelatin solution. Immediately remove gelatin solution and leave the covered plate to dry in the hood for 30 minutes.
    - For 6 well plate add 1.6 ml
    - For 12 well plate add 800 µL
    - For 24 well plate add 400 µL
    - For 48 well plate add 200 µL
3. Remove flask containing confluent ECs from incubator.
4. Aspirate supernatant and any cells in suspension. The supernatant and cells in suspension can be discarded. TIP: Be careful to not dislodge any cells adhered to the flask.
5. Gently rinse adherent cells twice with PBS to remove excess media.
    - For T75 Flask add 3 mL
    - For T25 Flask add 2 mL
6. Aspirate PBS from flask and discard.
7. Detach adherent cells from the flask by adding TrypLE Express.
    - For T75 Flask add 5 mL
    - For T25 Flask add 3 mL
8. Rock the flask back and forth to ensure TrypLE Express covers the entire bottom of the flask.
9. Incubate the cells with TrypLE Express for 3 minutes at 37°C to allow the cells to detach. During incubation, rock the flask back and forth about once per minute.

10. After incubation, check under microscope to determine if cells are detached. If so, proceed to next step. If not, continue to incubate at 37°C and check every 1 to 2 minutes.
11. Add complete RPMI and aspirate cells by gently pipetting.
    - For T75 Flask add 10 mL
    - For T25 Flask add 4 mL
12. Transfer cell suspension from flask to 15 mL centrifuge tube.
13. Centrifuge cells for 10 minutes at 300 rcf.
14. Remove the centrifuged cells and check for cell pellet.
15. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
16. Resuspend cell pellet in complete RPMI.
    - If T75 Flask was used, add 10 mL
    - If T25 Flask was used, add 4 mL
17. 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.
18. 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.
19. Remove the centrifuged cells and check for cell pellet.
20. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
21. Resuspend cells in complete RPMI to a density of 1 x 10^5 cells/mL.
22. Mix well by pipetting up and down 5 times. TIP: Be careful not to create bubbles.
23. Seed cell suspension into appropriately sized plate depending on volume of cell suspension. Add the cell suspension into coated wells on the previously prepared flat bottom plate.
    - For 6 well plate add 4 mL
    - For 12 well plate add 2 mL
    - For 24 well plate add 1 mL
    - For 48 well plate add 500 µL
24. Incubate plate overnight 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: 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 LPS as described in Chapter 4a.
2. Cell stimulation with TNF-α as described in Chapter 4b.

Chapter 4a: LPS Stimulation

Materials Required

LPS 1 mg/mL (-20°C)
Complete RPMI (37°C)
Plate Containing ECs from Chapter 3
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. Thaw a vial of stock 1 mg/mL LPS at room temperature.
2. Pipet up and down 5 times to ensure the LPS is well mixed.
3. Prepare a working stock of LPS. Add 1 µL of 1 mg/mL LPS to 100 µL complete RPMI. Final concentration is 10 µg/mL. CRITICAL: Working 10 µg/mL LPS stock should be made fresh. If there is any remaining 1 mg/mL LPS stock, discard and do not re-freeze.
4. Vortex LPS working stock for 10 seconds. TIP: Ensure contents are well-mixed.
5. Add 10 µL of 10 µg/mL LPS working stock for every 10 mL complete RPMI required into a 15 mL centrifuge tube. This yields a final concentration of 10 ng/ml. CRITICAL: Volume is dependent on number of wells.
    a. Use serological pipette to mix thoroughly. CRITICAL: Be careful not to create bubbles.
6. Take ECs from the incubator.
7. Aspirate supernatant and discard.
8. Add appropriate volume, according to Table 10, of complete RPMI/LPS to desired number of wells for stimulated condition on plate.
9. Add appropriate volume, according to Table 10, of complete RPMI to desired number of wells for unstimulated condition on plate.

Table 10: Plate and Volume for Endothelial Seeding

Plate	Volume
48 well plate	500 µL/well
24 well plate	1 mL/well
12 well plate	2 mL/well
6 well plate	4 mL/well

10. Incubate plate for 24 hours at 37°C, 5% CO_2.

    NOTE: Stimulation time may vary and is dependent upon the experimental design and/or passage of the cells.

Chapter 4b: TNF-α Stimulation

Materials Required

TNF-α 0.1 mg/mL (-20°C)
Complete RPMI (37°C)
Plate Containing ECs from Chapter 3
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. Thaw a vial of stock 0.1 mg/mL TNF-α at room temperature.
2. Pipet up and down 5 times to ensure the TNF-α is well mixed.
3. Prepare a working stock of TNF-α. Add 1 µL of 0.1 mg/mL TNF-α to 9 µL complete RPMI. Final concentration is 10 µg/mL. CRITICAL: Working 10 µg/mL TNF-α stock should be made fresh. If there is any remaining 0.1 mg/mL TNF-α stock, discard and do not re-freeze.
4. Vortex TNF-α working stock for 10 seconds. TIP: Ensure contents are well-mixed.
5. Add 10 µL of 10 µg/mL TNF-α working stock for every 10 mL complete RPMI required into a 15 mL centrifuge tube. This yields a final concentration of 10 ng/ml. CRITICAL: Volume is dependent on number of wells.
    a. Use serological pipette to mix thoroughly. CRITICAL: Be careful not to create bubbles.
6. Take ECs from incubator.
7. Aspirate supernatant and discard.
8. Add appropriate volume, according to Table 11, of complete RPMI/TNF-α to desired number of wells for stimulated condition on plate.

9. Add appropriate volume, according to Table 11, of complete RPMI to desired number of wells for unstimulated condition on plate.

Table 11: Plate and Volume for Endothelial Seeding

Plate	Volume
48 well plate	500 µL/well
24 well plate	1 mL/well
12 well plate	2 mL/well
6 well plate	4 mL/well

10. Incubate plate for 24 hours at 37°C, 5% CO_2.

NOTE: Stimulation time may vary and is dependent upon the experimental design and/or passage of the cells.

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

Stimulated or Unstimulated ECs in 6 Well, 12 Well. 24 Well, or 48 Well Plate
TrypLE Express (37°C)
15 mL Centrifuge Tubes (*Stimulated, Unstimulated*)
Lo-Bind Microcentrifuge Tubes (*Stain Master Mix, Stimulated, Unstimulated*)
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 plate with ECs from incubator.
4. Remove supernatant from wells* and discard.

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

5. Gently rinse adherent cells in each well twice with PBS to remove excess media.
    - For 6 well plate add 2 mL
    - For 12 well plate add 1 mL
    - For 24 well plate add 400 µL
    - For 48 well plate add 200 µL
6. Tilt plate and gently remove PBS. Discard rinse.
7. Detach adherent cells from the plate by adding TrypLE Express to each well.
    - For 6 well plate add 1 mL
    - For 12 well plate add 500 µL
    - For 24 well plate add 200 µL
    - For 48 well plate add 100 µL
8. Rock the plate back and forth to ensure TrypLE Express covers the entire bottom of the well.

9. Incubate the cells with TrypLE Express for 3 minutes at 37°C to allow the cells to detach. During incubation, rock the plate back and forth about once per minute.
10. After incubation, check under microscope to determine if cells are detached. If so, proceed to next step. If not, continue to incubate at 37°C and check every 1 to 2 minutes.
- 11. Rinse each well with complete RPMI. TIP: Rinse gently, be careful not to create bubbles.
    - For 6 well plate add 2 mL
    - For 12 well plate add 1 mL
    - For 24 well plate add 400 µL
    - For 48 well plate add 200 µL
- 12. Transfer stimulated cell suspension to a 15 mL centrifuge tube. Note: Pool wells if there are replicates into one tube.
13. Transfer unstimulated cell suspension to a 15 mL centrifuge tube. Note: Pool wells if there are replicates into one tube.
14. Centrifuge cells for 10 minutes at 300 rcf.
15. After cells are centrifuged, check for cell pellets.
- 16. 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.
17. Add 1 mL of PBS to both unstimulated and stimulated cell suspensions to dilute any remaining media and mix by pipetting up and down.
- CRITICAL: Failure to remove excess media will result in poor staining.
18. Transfer stimulated cell suspension from the 15 mL centrifuge tube to a Lo-Bind microcentrifuge tube.
19. Transfer unstimulated cell suspension from the 15 mL centrifuge tube to a Lo-Bind microcentrifuge tube.
20. Centrifuge cells for 10 minutes at 300 rcf.
21. After cells are centrifuged, check for cell pellets.
- 22. 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.
23. Gently remix stain master mix.
- CRITICAL: Failure to remix stain master mix will result in poor staining.
- 24. 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.
25. Incubate for 5 minutes at 37°C in the dark.
- 26. Gently pipet to mix the cell suspension 15 times. CRITICAL: Be careful to not create bubbles.
27. Incubate for an additional 5 minutes at 37°C in the dark.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

28. After incubation, add 5 times the volume of complete RPMI media. CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.
29. Incubate for 10 minutes at 37°C in the dark.
30. 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.
31. Centrifuge stained cells for 10 minutes at 300 rcf.
32. After cells are centrifuged, check for cell pellets.
33. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
34. Resuspend the cell pellets with complete RPMI to a cell density of 1 x 10^6 cells/mL.
35. Proceed to Chapter 7.

Chapter 7: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 5
Stained ECs 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 unstimulated cells 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. Resuspend stimulated cells 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.
4. Let IsoCode chips sit for one minute on a flat surface.
5. Check bottom of chip to ensure liquid has entered the chip. TIP: If liquid has not flowed, tap IsoCode chip on flat surface lightly.
6. When inserting IsoCode chip into the instrument, make sure the logo is facing up and towards you with the magnet facing the instrument. Take the blue film off while inserting each IsoCode chip into the instrument.

   NOTE: Please refer 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 EGM-2 MV media 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 Media (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 3×10^6 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 1×10^7 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.
12. Transfer cells into the 15 mL centrifuge tube(s) containing.

- 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 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. • Follow cell removal steps as highlighted in **Chapter 6** • Count within 15 minutes of staining the cells
Low quality cell count on chip *Stain Process related*	• Use of media other than the recommended media in protocol which could interact with cell stain • Use of stains not recommended in protocol • Recommended stain concentration, incubation time and/or incubation temperature not used • Cell stain 405 was stored prior to use • Media not completely removed from cell pellet prior to staining	• Prepare EGM-2 MV media following manufacturer's recipe • 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 6.16**

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** and **7.3** to avoid introduction of bubbles on chip
		• Ensure use of a sterile space to reduce introduction of potential contaminants. Use dedicated pipettes, tips, and tubes for sterile work. Pipette up and down gently and throughout protocol to reduce clumps.
		• 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 • Decreased viability due to cell shock • Recommended stimulation duration was not used	• After thaw, quickly transfer cells from DMSO to EGM-2 MV media to ensure viability of cells • Verify viability of cells is above 80% as stated in **Chapter 3** 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]) • Use stimulation timing in **Chapter 4**

Key: ● TIP, ● CRITICAL, ● OPTIONAL