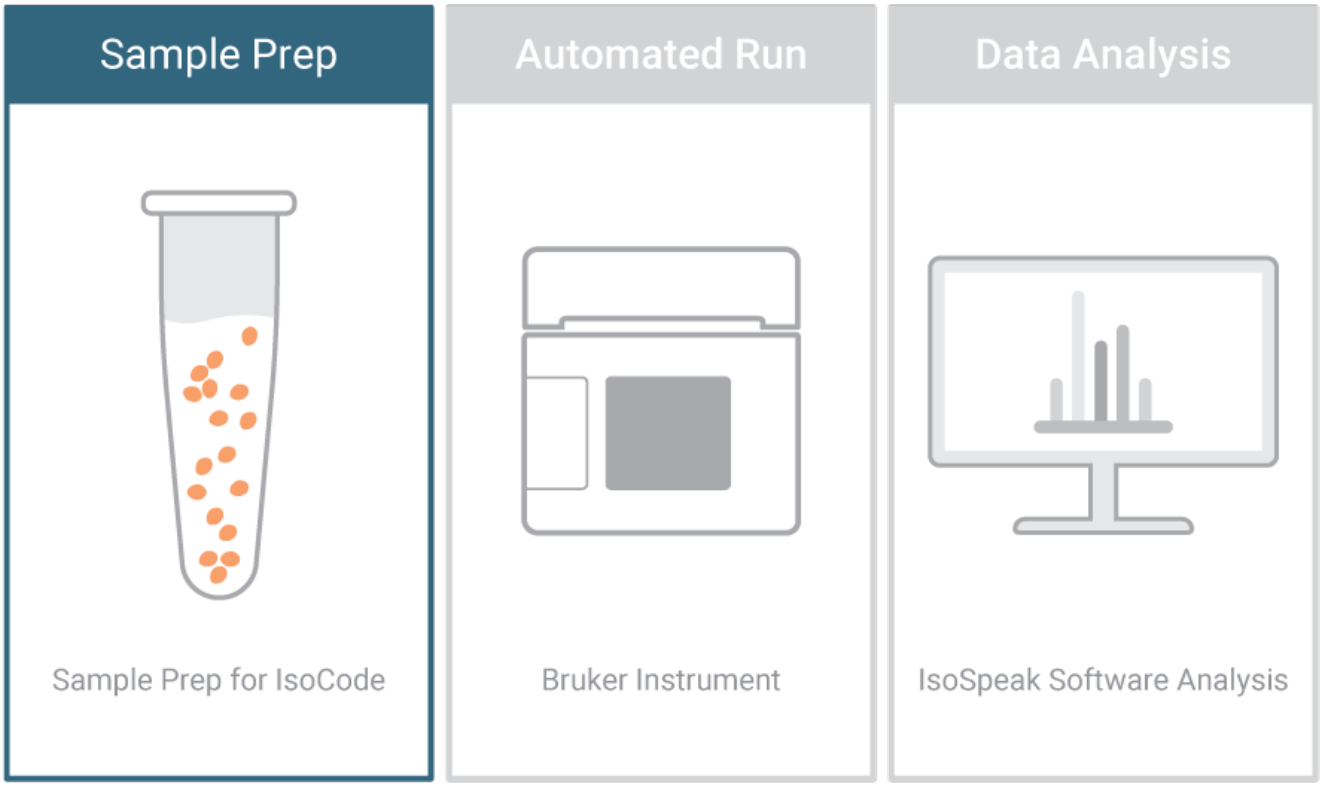
IsoCode Single-Cell Adaptive Immune: Human Induced Pluripotent Stem Cell Derived (iPSC-derived) Astrocytes Protocol

Ensure you achieve the maximum benefit from the Bruker systems and generate impactful data as quickly as possible



Key: ● TIP, ● CRITICAL, ● OPTIONAL

Contents

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A. Overview

Overview of Protocol

Day 1: Cryopreserved cells are thawed and cultured overnight.

Day 2: Media is changed, and cells are cultured for 7 days.

Day 8: Cell are lifted and re-plated. Culture for 7 days.

Day 15: Stimulation of iPSC-derived astrocytes for 24 hours.

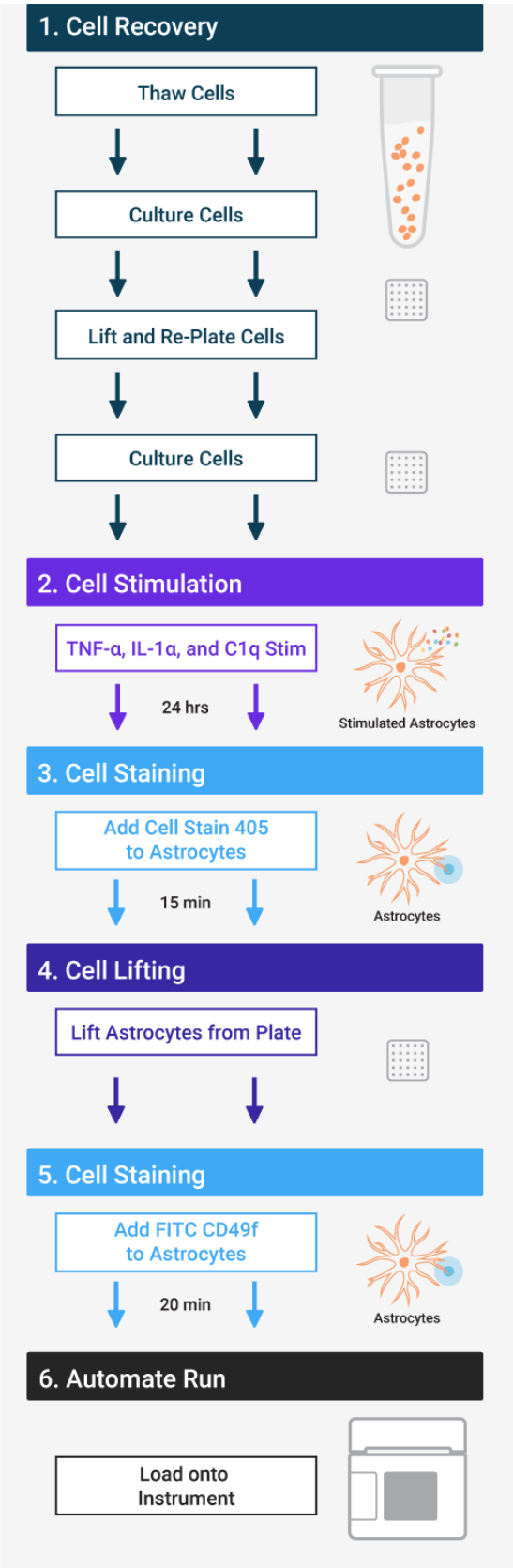
Day 16: Staining and Loading of iPSC-derived astrocytes onto IsoCode Chip.

NOTE:
This protocol outlines the standard method for thawing and culturing of human iPSC-derived astrocytes only and may not be valid for other species or cell types.

NOTE:
For brevity, when this protocol refers to astrocytes, this is meant to refer exclusively to human iPSC-derived astrocytes.

NOTE:
Validation of this protocol utilized Quick-Glia Astrocytes purchased from Elixirgen Scientific. Astrocytes procured from other vendors may be suitable, but would require separate testing as these haven't been utilized by Bruker.

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-46 REV 3.0

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
Centrifuge Tubes*	Polypropylene, 15 mL	VWR	CA62406-200
Centrifuge Tubes*	50 mL	VWR	21008-242
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
Astrocyte Basal Medium (Part of Astrocyte Medium Kit)	1x	ScienCell Research Laboratories	1801
Astrocyte Growth Supplement (AGS, Part of Astrocyte Medium Kit)	100x	ScienCell Research Laboratories	1801
Penicillin/Streptomycin Solution (Part of Astrocyte Medium Kit)	100x	ScienCell Research Laboratories	1801
Component P (Part of Quick-Glia Astrocyte Kit)	1x	Elixirgen Scientific	AS-SeV-CW50065
Geltrex LDEV-Free, h-ESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	100x	Gibco	A1413302
0.02 % Ethylenediaminetetraacetic acid (EDTA) in DPBS	0.5 mM	Millipore Sigma	E8008-100ML
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	Gibco	10010072
TrypLE Select Enzyme	1x	ThermoFisher	12563011
Recombinant Human IL-1α (Carrier-Free)	200 µg/mL	BioLegend	570002
Recombinant Human TNF-α (Carrier-Free)	200 µg/mL	BioLegend	570102
Complement Component C1q Native Protein	1 mg/mL	MyBioSource	MBS147305
FITC anti-human/mouse CD49f Stain	200 µg/mL	BioLegend	313606
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

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

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: Astrocyte Media with AGS (AGM) Recipe

- CRITICAL: AGM 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 60 mL	Vendor/Catalog
Penicillin/Streptomycin Solution	100x	1x	600 µL	ScienCell Research Laboratories/1801
Astrocyte Growth Supplement (AGS)	100x	1x	600 µL	ScienCell Research Laboratories/1801
Basal Medium	1x	1x	58.8 mL	ScienCell Research Laboratories/1801

Note | Warm all three components prior to preparing AGM media for 1 hour at room temperature away from light. Sterile-filter through 0.20 µm filter before use. Store AGM Media at 4°C and warm up at room temperature for 30 to 40 minutes prior to use.

Table 8: Astrocyte Media (AM) Recipe

- CRITICAL: AM 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 100 mL	Vendor/Catalog
Penicillin/Streptomycin Solution	100x	1x	1 mL	ScienCell Research Laboratories/1801
Basal Medium	1x	1x	99 mL	ScienCell Research Laboratories/1801

Note | Warm components prior to preparing AM media for 1 hour at room temperature away from light. Sterile-filter through 0.20 µm filter before use. Store AM Media at 4°C and warm up at room temperature for 30 to 40 minutes prior to use.

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

- CRITICAL: IL-1α 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 10 mL	Vendor/Catalog
Recombinant Human IL-1α (Carrier-Free)	200 µg/mL	1 µg/mL	50 µL	BioLegend/570002
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	1x	9.95 mL	Gibco/10010072

- CRITICAL: Prepare 50 µL aliquots and freeze at -20°C for no longer than 3 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: Working Stock of TNF-α (10 µg/mL) 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
Recombinant Human TNF-α (Carrier-Free)	200 µg/mL	1 µg/mL	50 µL	BioLegend/570102
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	1x	950 µL	Gibco/10010072

- CRITICAL: Prepare 50 µL aliquots and freeze at -20°C for no longer than 3 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 11: Working Stock of C1q (100 µg/mL) Recipe

- CRITICAL: C1q 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 2 mL	Vendor/Catalog
Complement Component C1q Native Protein	1 mg/mL	100 µg/mL	200 µL	MyBioSource/MBS147305
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	1x	1.8 mL	Gibco/10010072

- CRITICAL: Prepare 50 µL 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.

Additional Reagent to Be Prepared

NOTE: Geltrex should be thawed at 4°C overnight and aliquoted upon receipt from vendor. Keep Geltrex and aliquots on ice during the process. 100 µL aliquots are recommended and storage is at -80°C for up to 3 years.

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: Coat Culture Plates with Geltrex

Materials Required

Geltrex (-80°C)
Sterile 1X PBS (Room Temperature)
2 x 15 mL Centrifuge Tubes (*1X PBS/Geltrex, AGM Media*)
6 Well Plate
AGM Media (4°C)

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

Methods

1. Aliquot 10 mL of 1X PBS into a 15 mL centrifuge tube. Prechill 1X PBS for 1 hour at 4°C.
2. Thaw a vial of Geltrex at ambient temperature.

3. Prepare a Geltrex/PBS mixture.
    a. Mix thawed Geltrex by gently pipetting up and down.
    b. Remove prechilled 1X PBS from 4°C.
    c. Add 100 µL of Geltrex to prechilled 1X PBS.
    d. Use serological pipette to mix thoroughly.

    NOTE: Keep 15 mL centrifuge tube with Geltrex/PBS on ice. Discard remaining Geltrex – aliquots are single use only and cannot be refrozen.
4. Add 1.5 mL of Geltrex/PBS mixture to each well on a 6 well flat bottom plate.
5. Incubate covered plate for 1 hour at 37°C, 5% CO_2.
6. While plate is incubating, aliquot 7 mL of AGM media into a 15 mL centrifuge tube. Warm AGM media for 30 to 40 minutes at room temperature.
7. Remove plate from incubator and aspirate the majority of the Geltrex/PBS mixture from each well with a pipette. NOTE: Do not remove all of the Geltrex/PBS mixture. Leave approximately 500 µL per well.
8. Add 1 mL of AGM media to each well.
9. Incubate plate at 37°C, 5% CO_2 until cells are ready for plating.

Chapter 3: Recovery of Cryopreserved Cells

Materials Required

AGM Media (4°C)
15 mL Centrifuge Tube
Cryopreserved Astrocytes
Coated 6 Well Plate from Chapter 2
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. Warm AGM media for 30 to 40 minutes at room temperature.
2. Pipette 4.5 mL of AGM media into a 15 mL centrifuge tube, labeled *Thawed Astrocytes*.
- 3. Using proper PPE, remove cells from liquid nitrogen storage and thaw cells. TIP: Be careful of contamination.
4. 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.
5. 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.

6. Using a P1000 set to 1 mL, take 500 µL of AGM media and add AGM media dropwise into the vial. NOTE: Add approximately 1 drop every 2 seconds. Use the same pipette tip for steps 6 through 12.
7. Gently pipette the cells up and down one time.
- 8. Slowly pipette thawed cells into 4 mL of AGM media in 15 mL centrifuge tube, labeled *Thawed Astrocytes*. TIP: Insert tip into AGM media when pipetting, be careful to not create bubbles.
9. Take 1 mL of the cell/AGM media mixture.
10. Pipette into original thawed cell vial and pipette up and down 3 times to recover additional thawed cells. TIP: Insert tip into AGM media, be careful to not create bubbles.
11. Draw up cell/AGM media mixture and pipette back into the 15 mL centrifuge tube, labeled *Thawed Astrocytes*.
- 12. Mix well by pipetting up and down 3 times. TIP: Insert tip into AGM media and pipette gently up and down. Be careful to not create bubbles.
13. Centrifuge cells for 4 minutes at 200 rcf.
14. After cells are centrifuged, check for cell pellet.
- 15. Aspirate the majority of the supernatant. Leave a small volume of supernatant to cover cell pellet. TIP: Be careful not to aspirate cell pellet.
16. Tap the side of the 15 mL centrifuge tube 10 times to break up cell pellet.
17. Resuspend cells in 1 mL of fresh AGM media.
- a. Using a P1000, mix well by pipetting up and down 3 times. NOTE: Do not pipette more than 3 times. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
- 18. 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.
- 19. Centrifuge cells for 4 minutes at 200 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.
20. After cells are centrifuged, check for cell pellet.
- 21. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
22. Resuspend cell pellet with AGM media to a cell density of 5 x 10^5 cells/mL.
23. Remove previously prepared Geltrex coated 6 well plate from incubator.
- 24. Plate 500 µL of cell suspension per Geltrex coated well in the 6 well plate. NOTE: Total volume per well is now 1.5 mL containing 2.5 x 10^5 cells.
25. Gently rock the plate 5 times in a cross formation. NOTE: Rock the plate back and forth and then side to side.
26. Incubate plate for 24 hours at 37°C, 5% CO_2.

Chapter 4: Media Change

Materials Required

AGM Media (4°C)
Component P (-20°C)
6 Well Plate Containing Astrocytes from Chapter 3
50 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. Prepare AGM media with Component P.
    a. Aliquot 40 mL of AGM Media into a 50 mL centrifuge tube.
    b. Warm AGM media for 30 to 40 minutes at room temperature.
    c. Remove 2 vials of Component P from -20°C and thaw at room temperature for 20 to 30 minutes.
    d. Once AGM Media and Component P have come to room temperature, add 20 µL of Component P to AGM media in the 50 mL centrifuge tube.
2. Take cells out of the incubator.
3. Aspirate the majority of the AGM media from one well leaving approximately 500 µL. Add 2 mL of AGM media with Component P to the well. NOTE: Change media one well at a time.
4. Repeat step 3 until media has been changed for all wells.
5. Incubate plate for 2 days at 37°C, 5% CO_2. Store remaining AGM media with Component P at 4°C for up to 1 week.

Chapter 5: Cell Culture and Media Change

Materials Required

AGM Media with Component P (4°C)
6 Well Plate Containing Astrocytes

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

Methods

NOTE: Complete this Chapter every 2 to 3 days while culturing the astrocytes.

1. Warm AGM media with Component P for 30 to 40 minutes at room temperature.
2. Take cells out of the incubator.
3. Aspirate the majority of the AGM media from one well leaving approximately 500 µL. Add 2 mL of AGM media with Component P to the well. NOTE: Change media one well at a time.

4. Repeat step 3 until media has been changed for all wells.
5. Incubate plate at 37°C, 5% CO_2 until additional media change is required or confluency is reached. NOTE: Typically, 7 days is required to reach confluency.
6. Once cells have reached confluency, cells can be passaged or cryopreserved for later use. Proceed to Chapter 6 to prepare for passaging cells for continuation of this protocol.

Chapter 6: Coat Culture Plates with Geltrex

Materials Required

Geltrex (-80°C) Sterile 1X PBS (Room Temperature) 2 x 15 mL Centrifuge Tubes (*1X PBS/Geltrex, AGM Media*) 6 Well Plate AGM Media (4°C)

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

Methods

NOTE: If sufficient cells are obtained in Chapter 7, preparing two plates is recommended. The following steps describe coating one plate. Two plates can be prepared in parallel. If a second plate is prepared and not used, it can be stored wrapped in parafilm for up to 1 week at 4°C. If removing a plate from 4°C, place at room temperature for 1 hour prior to use.

1. Aliquot 10 mL of 1X PBS into a 15 mL centrifuge tube. Prechill 1X PBS for 1 hour at 4°C.
2. Thaw a vial of Geltrex at ambient temperature.
3. Prepare a Geltrex/PBS mixture.
    a. Mix thawed Geltrex by gently pipetting up and down.
    b. Remove prechilled 1X PBS from 4°C.
    c. Add 100 µL of Geltrex to prechilled 1X PBS.
    d. Use serological pipette to mix thoroughly.

    NOTE: Keep 15 mL centrifuge tube with Geltrex/PBS on ice. Discard remaining Geltrex – aliquots are single use only and cannot be refrozen.
4. Add 1.5 mL of Geltrex/PBS mixture to each well on a 6 well flat bottom plate.
5. Incubate covered plate for 1 hour at 37°C, 5% CO_2.
6. While plate is incubating, aliquot 7 mL of AGM media into a 15 mL centrifuge tube. Warm AGM media for 30 to 40 minutes at room temperature.
7. Remove plate from incubator and aspirate the majority of the Geltrex/PBS mixture from each well with a pipette. NOTE: Do not remove all of the Geltrex/PBS mixture. Leave approximately 500 µL per well.

8. Add 1 mL of AGM media to each well.
9. Incubate plate at 37°C, 5% CO_2 until cells are ready for plating.

Chapter 7: Cell Lifting and Plating

Materials Required

AGM Media (37°C)
TrypLE Select (Room Temperature)
EDTA (4°C)
Confluent Astrocytes
15 mL Centrifuge Tubes
1.5 mL Lo-Bind Microcentrifuge Tube (*Cell Count*)
Geltrex Coated 6 Well Plate from Chapter 6

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

Methods

1. Warm AGM media for 30 to 40 minutes at room temperature.
2. Prepare a TrypLE Select and EDTA 1:1 mixture in a 15 mL centrifuge tube.
    a. Add 2 mL of TrypLE Select to a 15 mL centrifuge tube.
    b. Add 2 mL of EDTA to a 15 mL centrifuge tube.
    c. Mix well 5 times with a 5 mL serological pipette.
3. Take astrocytes out of incubator.
4. Aspirate AGM media with component P from one well with a P1000. Detach adherent cells from plate by gently adding 500 µL of the TrypLE Select/EDTA mixture to the well.
5. Repeat step 4 until all wells contain the TrypLE Select/EDTA mixture.
6. Incubate the cells with the TrypLE Select/EDTA mixture for 5 minutes at 37°C to allow cells to detach.
7. Rock the plate back and forth.
8. Observe the cells under the microscope to ensure cells are starting to detach from plate.
9. After incubation, add 500 µL of AGM media to each well. Pipette up and down to rinse the entire surface of each well and to lift the cells from the plate.
10. Collect cells/TrypLE Select/EDTA/AGM media mixture and transfer to a 15 mL centrifuge tube.
11. Gently rinse each well with 500 µL of AGM media.
12. Collect cells/AGM media mixture and transfer to the 15 mL centrifuge tube.
13. 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.

14. Centrifuge cells for 5 minutes at 200 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.
15. After cells are centrifuged, check for cell pellet.
16. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to remove last bit of supernatant.
17. Resuspend cell pellet with AGM media to a cell density of 9 x 10^4 cells/mL.
18. Remove previously prepared Geltrex coated 6 well plate from incubator.
19. Plate 1 mL of cell suspension per Geltrex coated well in the 6 well plate. NOTE: Total volume per well is 2.5 mL. If there is remaining cell suspension after plating 6 wells, it is recommended to use a second plate to maximize cell numbers.
20. Gently rock the plate 5 times in a cross formation. NOTE: Rock the plate back and forth and then side to side.
21. Move plate to incubator for cell culture at 37°C, 5% CO_2.

Chapter 8: Cell Culture and Media Change

Materials Required

AGM Media (4°C)
6 Well Plate Containing Astrocytes

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

Methods

NOTE: Complete this Chapter every 2 to 3 days while culturing the astrocytes.

1. Warm AGM media for 30 to 40 minutes at room temperature.
2. Take cells out of the incubator.
3. Aspirate the majority of the AGM media from one well leaving approximately 500 µL. Add 2 mL of AGM media to the well. NOTE: Change media one well at a time.
4. Repeat step 3 until media has been changed for all wells.
5. Incubate plate at 37°C, 5% CO_2 until additional media change is required or confluency is reached. NOTE: Typically, 7 days is required to reach confluency.
6. Once cells have reached confluency, proceed to Chapter 9 (Cell Stimulation).

   NOTE: At minimum one 6 well plate with confluent astrocytes is required to proceed to Chapter 9. It is recommended to use two 6 well plates.

Chapter 9: Cell Stimulation

Materials Required

AM Media (4°C)
15 mL Centrifuge Tubes
TNF-α 10 µg/mL (-20°C)
IL-1α 1 µg/mL (-20°C)
C1q 100 µg/mL (-20°C)
Confluent Astrocytes

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

Methods

NOTE: The steps below describe working with one plate. If working with two plates, conduct steps in parallel for each plate and scale up volumes as required.

1. Warm AM media for 30 to 40 minutes at room temperature. NOTE: This media does not contain AGS.
2. Thaw a vial of stock 10 µg/mL TNF-α, a vial of 1 µg/mL IL-1α and a vial of stock 100 µg/mL C1q at ambient temperature.
3. Vortex TNF-α, IL-1α, and C1q for 5 seconds.
- 4. Spin TNF- α, IL-1α, and C1q in a mini centrifuge for 10 seconds. TIP: Ensure that contents are all in the bottom of the vial.
- 5. Prepare "Stimulated AM Media" mixture. CRITICAL: Volume is dependent on the number of wells that will be stimulated.
    a. Aliquot 10 mL of AM media into a 15 mL centrifuge tube labeled "Stimulated AM Media".
    b. Add 30 µL of TNF-α (10 µg/mL) to the AM media. Final concentration is 30 ng/mL.
    c. Add 30 µL of IL-1α (1 µg/mL) to the AM media. Final concentration is 3 ng/mL.
    d. Add 40 µL of C1q (100 µg/mL) to the AM. Final concentration is 400 ng/mL.
    e. Use serological pipette to mix thoroughly.
- CRITICAL: Discard remaining/unused TNF-α, IL-1α, and C1q – aliquots are single use only and cannot be refrozen.
6. Prepare AM Unstimulated media.
    a. Aliquot 5 mL of AM media into a 15 mL centrifuge tube labeled "AM Media Unstimulated"—set this AM media aside as it will serve as the AM media used for the unstimulated (negative control) condition.
7. Take cells out of the incubator.
- 8. Aspirate media using a P1000 from one well. CRITICAL: Be careful not to disturb adherent astrocytes on plate.

9. Add 2 mL of "Stimulated AM Media" or "AM Media Unstimulated" to well.

- a. For a well that will be used for the unstimulated condition, add 2 mL of "AM Media Unstimulated". NOTE: Gently pipette media down the wall of the well, not directly onto the cells, to avoid displacing adherent cells. TIP: Setup at least one well on the 6 well plate for the unstimulated condition.

b. For a well that will be used for the stimulated condition, add 2 mL of "Stimulated AM Media". NOTE: Gently pipette media down the wall of the well, not directly onto the cells, to avoid displacing adherent cells.

NOTE: Change media one well or flask at a time. For a 6 well plate, it is suggested to add "AM Media Unstimulated" to 2 wells and "Stimulated AM Media" to 4 wells.

10. Repeat steps 8 and 9 until media has been changed for all wells.

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

Chapter 10: Cell Staining (Violet) and Cell Lifting

Materials Required

Stimulated or Unstimulated Astrocytes in 6 Well Plate
15 mL Centrifuge Tubes (*Stimulated, Unstimulated, Stain Master Mix, TrypLE Select/EDTA Mixture*)
Sterile 1X PBS (Room Temperature)
TrypLE Select (Room Temperature)
EDTA (4°C)
AM Media (4°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

NOTE: The steps below describe working with one plate. If working with two plates, conduct steps in parallel for each plate and scale up volumes as required.

1. Warm AM media for 30 to 40 minutes at room temperature. NOTE: Warm enough media to complete Chapters 10 and 12.

2. 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.

3. Prepare stain master mix by diluting 14 µL of cell stain 405 into 7 mL of 1X PBS in a 15 mL centrifuge 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 number of wells, 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 5 mL serological pipette, 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.**
4. Remove 6 well plate with astrocytes from incubator.
5. From one well, remove supernatant with a P1000 pipette and discard.* CRITICAL: Be careful not to disturb adherent astrocytes plate. Ensure all media is removed.

    *NOTE: Supernatants may be stored at -80°C for bulk assay.
6. Add 1 mL of 1X PBS to well. **NOTE: Gently pipette 1X PBS down the wall of the well, not directly onto the cells, to avoid displacing adherent cells.**

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

7. Repeat steps 5 and 6 until all wells contain 1X PBS.
8. Gently remix stain master mix.

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

9. Remove 1X PBS wash from one well with a P1000 pipette.
10. Add 1 mL of stain master mix to well. **NOTE: Gently pipette stain master mix down the wall of the well, not directly onto the cells, to avoid displacing adherent cells.** CRITICAL: Gently remix master mix if it has been sitting for longer than a few minutes.
11. Repeat steps 9 and 10 until stain master mix has been added to all wells.
12. Incubate for 15 minutes at 37°C in the dark.
13. While cells incubate, prepare a TrypLE Select and EDTA 1:1 mixture in a 15 mL centrifuge tube.
    a. Add 2 mL of TrypLE Select to a 15 mL centrifuge tube.
    b. Add 2 mL of EDTA to a 15 mL centrifuge tube.
    c. Mix well 5 times with a 5 mL serological pipette.
14. After incubation, add 1 mL of AM media to each well.
15. Aspirate stain master mix/AM media from one well with a P1000. Detach adherent cells from plate by gently adding 500 µL of the TrypLE Select/EDTA mixture to the well.
16. Repeat step 15 until all wells contain the TrypLE Select/EDTA mixture.
17. Incubate the cells with the TrypLE Select/EDTA mixture for 5 minutes at 37°C to allow cells to detach.
18. Rock the plate back and forth.
19. Observe the cells under the microscope to ensure cells are starting to detach from plate.

20. After incubation, add 500 µL of AM media to each well. Pipette up and down to rinse the entire surface of each well and to lift the cells from the plate.
21. Collect cells/TrypLE Select/EDTA/AM media mixture and transfer to a 15 mL centrifuge tube.
    a. From wells containing unstimulated astrocytes, transfer mixture to a 15 mL centrifuge tube labeled "Unstimulated Astrocytes".
    b. From wells containing stimulated astrocytes, transfer mixture to a 15 mL centrifuge tube labeled "Stimulated Astrocytes".
22. Gently rinse each well with 500 µL of AM media.
23. Collect cells/AM media mixture and transfer to a 15 mL centrifuge tube.
    a. From wells containing unstimulated astrocytes, transfer mixture to 15 mL centrifuge tube labeled "Unstimulated Astrocytes".
    b. From wells containing stimulated astrocytes, transfer mixture to 15 mL centrifuge tube labeled "Stimulated Astrocytes".
24. Centrifuge cells for 5 minutes at 200 rcf.
25. Proceed immediately to the next chapter.

Chapter 11: 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 13).
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 12: Surface Marker-Specific Staining (Blue)

Materials Required

Cells Stained with Cell Stain 405 from Chapter 10
AM Media (Room Temperature)
FITC anti-human/mouse CD49f (4°C)
Lo-Bind Microcentrifuge Tubes (*Stain Master Mix, 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. Spin tube of FITC anti-human/mouse CD49f in a mini centrifuge for 10 seconds to collect stain at the bottom of the tube.
2. Prepare FITC anti-human/mouse CD49f stain master mix by diluting 5 µL of FITC anti-human/mouse CD49f into 95 µL of AM media in a Lo-Bind microcentrifuge tube (1:20 final dilution) per well used on a 6 well plate. With the same pipette tip, pipette up and down 10 times to ensure all CD49f-FITC stain has been released. 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. After cells are centrifuged, check for cell pellets.
4. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
5. For every well used on a 6 well plate, add 100 µL of FITC CD49f master mix to each cell suspension tube. CRITICAL: Pipet to mix the cells 15 times. Be careful to not create bubbles.
6. Incubate for 20 minutes at room temperature in the dark.
7. After incubation, add 100 µL of AM media per well used to each sample tube. TIP: Mix gently, be careful not to create bubbles.
8. 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.
9. Centrifuge stained cells for 5 minutes at 200 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. Resuspend the cells with AM media to a cell density of 1 x 10^6 cells/mL. Proceed to Chapter 13.

Chapter 13: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 11
Stained Astrocytes 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 astrocytes 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 astrocytes 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 AGM media or AM 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]

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 3**) • 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 **Chapters 7 and 10** • 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 cells prior to staining	• Use AGM or AM media following recipes in **Tables 7 and 8** • Use Bruker validated stains (**Table 4: Cell Staining Reagents**) • Follow staining steps as highlighted in **Chapters 10 and 12** • Use only freshly prepared cell stain 405 per **Chapter 10** • Ensure all media is removed from cells in **step 10.5**

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 **13.2** and **13.3** 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 13**)
		• 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 • Decreased viability due to cell shock	• After thaw, quickly transfer cells from DMSO to AGM media to ensure viability of cells • Use reagents at recommended temperatures (i.e. always use warmed media [room temperature])

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Stimulation step related*	• Recommended TNF-α, IL-1α and C1q stimulation concentration was not used • Recommended TNF-α, IL-1α and C1q stimulation duration was not used • Expired TNF-α, IL-1α or C1q used	• Use TNF-α, IL-1α and C1q concentrations listed in Chapter 5 • Use TNF-α, IL-1α and C1q stimulation duration listed in Chapter 5 • Do not use TNF-α stock older than 3 months • Do not use IL-1α stock older than 3 months • Do not use C1q stock older than 6 months • Use required vendors as listed in Table 3