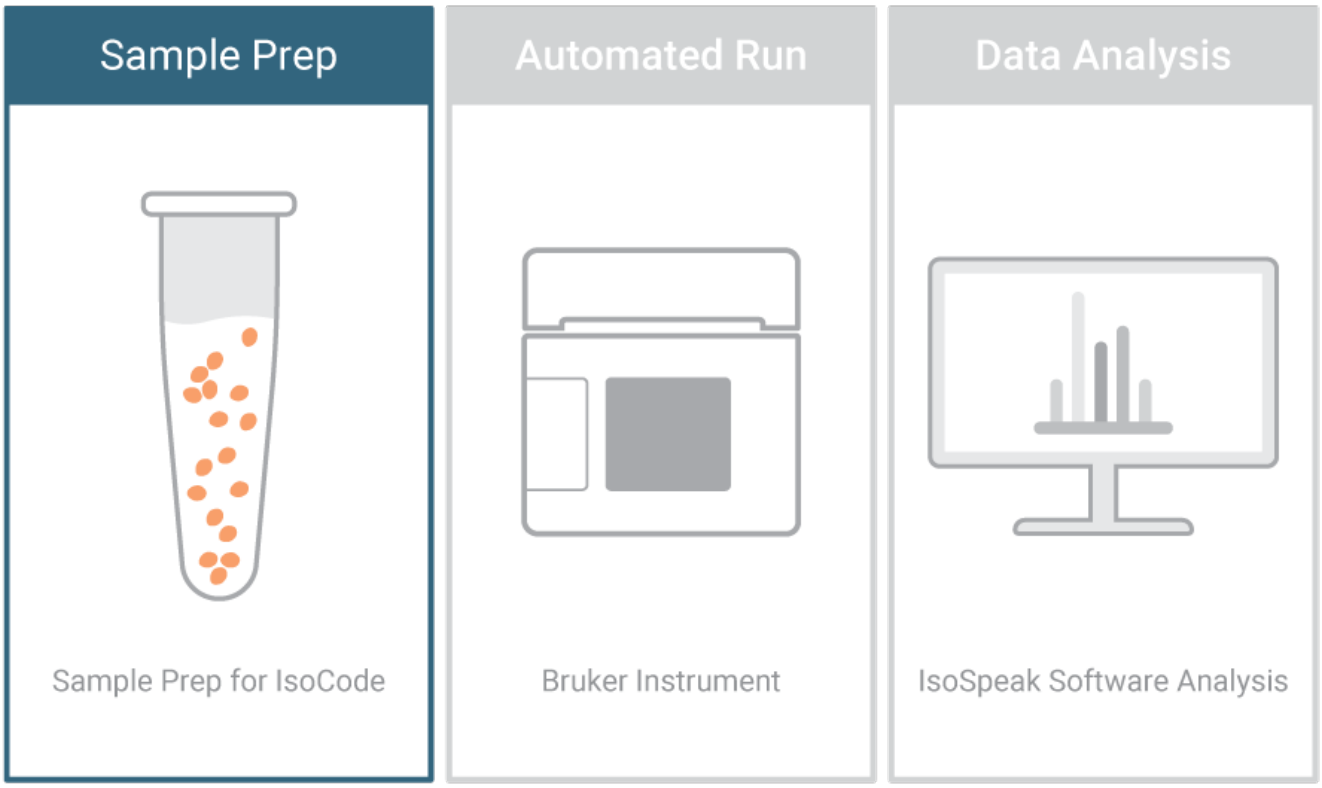
Single-Cell Tumor Signaling: HeLa Cell Protocol

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



Key: ● TIP, ● CRITICAL, ● OPTIONAL

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A. Overview

Overview of Protocol

Day 1-3: Cryopreserved HeLa cells are thawed and cultured for 72 hours.

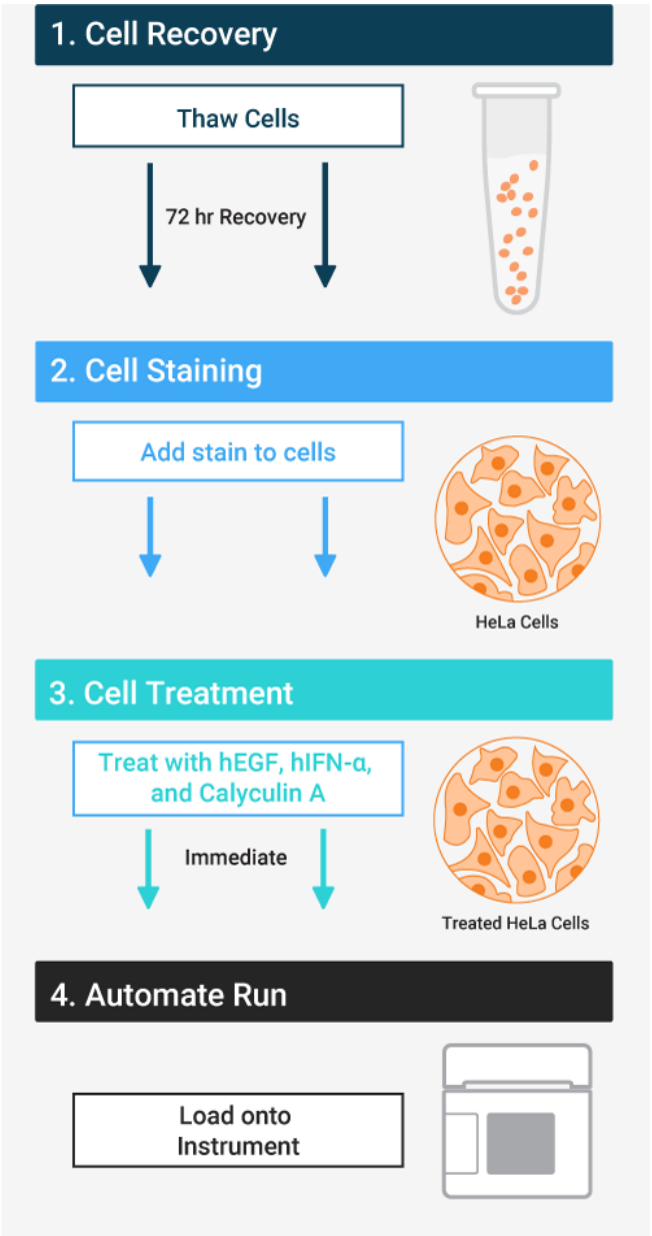
Day 4: Staining and stimulating of HeLa cells followed by immediate loading onto IsoCode Chip.

NOTE:
This protocol outlines the standard method for thawing and culturing of human HeLa 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.

NOTE:
This protocol focuses on HeLa cell handling and signaling stimulation/repression with hEGF, hIFN-α, and Calyculin A. If you are using a different cell type or have a different drug treatment paradigm that you would like to analyze, please work with your local Field Application Scientist (FAS) to plan your experiment. Over 20 cell lines have been tested on the Single-cell Signaling Solution.

Cell Line	Tumor Type
HeLa	Cervical Adenocarcinoma
Primary cell lines	Melanoma
U87	Glioblastoma
Cal27, UMSCC47, HN5	Squamous Cell Carcinoma of the Tongue
Inducible shRNA knockdowns	Lung Adenocarcinoma
Contact your FAS to plan an experiment with your cell line	



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Safety Warnings

- Read MSDS documents of all materials prior to use.

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

Required Reagents, Consumables and Equipment

Table 1: Required Reagents and Consumables Provided by Bruker

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

IsoCode Kit Components

IsoLight IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 1.5 mL Tube A: Cocktail A in Micro-Tube (Green Cap)
- 50 mL Tubes containing Reagents 1, 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 Tube A: Cocktail A in Micro-Tube (Green 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 chip kits
- 50 mL tube containing reagent 2 for IsoLight or 50 mL tube containing reagent 5 for IsoSpark

NOTE: Ensure boxes of IsoCode chips and reagent box have the same kit ID on each label.

Table 2: Required Consumables Not Supplied by Bruker

Consumable	Type	Source	Catalog Number
T25 Flask	N/A	Corning	353108
T75 Flask	N/A	Corning	430641U
6 Well Plate Flat Bottom	N/A	Corning	353046
Centrifuge Tubes*	Polypropylene, 15 mL	VWR	62406-200
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
70 µm Cell Strainer	N/A	Corning	352350
Syringe with BD Luer-Lok Tip	10 mL	VWR	75846-756
0.2 µm Syringe Filter with Acrylic Housing	N/A	VWR	28145-501
Fisherbrand Disposable PES Filter Units (0.20 µm)	500 mL	Fisher Scientific	FB12566504

*Bruker strongly recommends that low protein binding centrifuge tubes are used for cell culture work to ensure optimal cell pelleting.

Table 3: Required* Reagents Not Supplied by Bruker

Reagent	Stock Concentration	Source	Catalog Number
RPMI	1x	Fisher	MT10040CV
Penicillin-Streptomycin-Neomycin Solution Stabilized	100x	Sigma	P4083-100mL
Glutamax	100x	Thermo	35050061
FBS	1x	Sigma	F2442-6X500mL
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	Gibco	10010072
Trypan Blue	0.4%	Gibco	15250-061
Reagent Alcohol 70%	N/A	Lab Grade	N/A
CellTrace Far Red Cell Proliferation Kit	N/A	Invitrogen	C34564
DMSO (Part of Far Red Kit)	N/A	Invitrogen	C34564
hEGF	N/A	Cell Signaling Technology (CST)	72528S
TrypLE Express Enzyme	1X	ThermoFisher/Invitrogen	12604013
hIFN-α	N/A	Cell Signaling Technology (CST)	36000S
Calyculin A	N/A	Cell Signaling Technology (CST)	9902S
Protease Phosphatase Inhibitor	100X	Cell Signaling Technology (CST)	5872
Dimethyl Sulfoxide (DMSO)	N/A	Sigma-Aldrich	D8418

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

Table 4: Required Equipment

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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	02-671-51B
Hemocytometer Cover Glass	(Fisher) Hauser Levy	02-671-53

Table 5: 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 6: 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 7: Recipe for hEGF

Ingredient	Stock Concentration	Final Concentration	Amount for 1 mL	Vendor/Catalog
hEGF, lyophilized powder	N/A (100 µg)	100 µg/mL	Entire Vial	CST/72528S
Sterile Deionized Water	1X	1X	1 mL	N/A

- CRITICAL: Prepare 25 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 8: Recipe for hIFN-α

Ingredient	Stock Concentration	Final Concentration	Amount for 200 µL	Vendor/Catalog
hIFN-α	N/A (20 µg)	100 µg/mL	Entire Vial	CST/36000S
Sterile Deionized Water	1X	1X	200 µL	N/A

- CRITICAL: Prepare 5 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.

Additional Reagent to Be Prepared

NOTE: Calyculin A should be reconstituted in 1 mL DMSO for 10 µM stock and then aliquoted upon receipt from vendor. 5-10 µL aliquots are recommended and storage is at -20°C for up to 3 months.

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: Cocktail A in Micro-Tube (Green Cap)
- 50 mL Tubes Containing Reagents 1, 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 Tube A: Cocktail A in Micro-Tube (Green 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 chip kits
- 50 mL tube containing reagent 2 for IsoLight or 50 mL tube containing reagent 5 for IsoSpark

NOTE: Ensure boxes of IsoCode chips and reagent box have the same kit ID on each label.

Chapter 2: Recovery of Cryopreserved Cells

Materials Required

- Complete RPMI (37°C)
- Cryopreserved HeLa Cells
- 15 mL Centrifuge Tube
- Lo-Bind Microcentrifuge Tube for Cell Count
- Plate and/or Flask
  - For 2-5 M cells, T75 Flask
  - For 1-2 M cells, T25 Flask
  - For < 1 M cells, 6 Well Plate

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

Methods

1. Pipette 5 mL of complete RPMI into a 15 mL centrifuge tube, labeled *Thawed HeLa*.
2. Using proper PPE, remove cells from liquid nitrogen storage and thaw cells. TIP: Be careful of contamination.
3. Quickly move vials into a water bath (37°C) to thaw. While thawing, swirl the vial in the water until a single ice crystal remains in the vial. Be sure to prevent (to the best of your ability) any of the water from the water bath from getting under the cap and into the sample.
4. When the sample is nearly thawed, remove the vial and immediately spray vial with 70% alcohol before bringing into the hood. It is important to allow the alcohol to evaporate before opening the vial.
5. Slowly pipette thawed cells into 5 mL of complete RPMI in 15 mL centrifuge tube, labeled *Thawed HeLa*. TIP: Insert tip into complete RPMI when pipetting, be careful to not create bubbles.
6. Take 1 mL of complete RPMI and pipette into original thawed cell vial. Rinse inside the vial with the complete RPMI to recover additional thawed cells. TIP: Insert tip into complete RPMI, be careful to not create bubbles.
7. Draw up cell/complete RPMI mixture and pipette into the 15 mL centrifuge tube, labeled *Thawed HeLa*. TIP: Insert tip into complete RPMI and pipette gently up and down. Be careful to not create bubbles.
8. Centrifuge cells for 10 minutes at 300 rcf.
9. After cells are centrifuged, check for cell pellet.
10. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to remove last bit of supernatant.
11. Resuspend cell pellet in 1 mL of fresh complete RPMI.
    a. Mix well to resuspend. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
    b. Take 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.
12. Slowly add additional complete RPMI to a final concentration of 1 x 10^5 cells/mL.
13. Mix with serological pipette. TIP: Gently pipet up and down 3-5 times, be careful to not create bubbles.
14. Transfer cell suspension to flask or plate. TIP: Slowly pipette down the side of the flask as to not create bubbles.
15. Spread out cell suspension by rocking flask or plate carefully to fully cover the bottom of the container. TIP: Be careful to not make bubbles.
16. Move to incubator for 72-hour recovery at 37°C, 5% CO_2 to ensure that cells are fully recovered from freezing. Cells should be growing in log phase and at ~70-80% confluency before proceeding.
17. After 72 hours, observe flask or plate under light microscope and ensure cells are well-adhered and between 70 and 80% confluent. If so, continue to Chapter 3 and if not, continue culturing until desired confluency is reached.

Chapter 3: Staining and Lifting Recovered Cells

Materials Required

Complete RPMI (37°C)
TrypLE Express Enzyme (Room Temperature)
CellTrace Far Red (-20°C)
DMSO (-20°C)
Sterile 1X PBS (Room Temperature)
15 mL Centrifuge Tubes (*Stain Master Mix, Stimulated, Unstimulated*)
Recovered Cells from Chapter 2
2 x Lo-Bind Microcentrifuge Tube (*Working Stock, Cell Count*)

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

- CRITICAL: Before starting this section, ensure that you have enough time to complete Chapters 3 – 6 as you cannot stop. This is due to the transient nature of protein phosphorylation. Estimated time is 90-120 minutes depending on the number of samples and familiarity with the protocol.

  It is also critical to begin chip and lysis buffer thawing (Reagent 2 for IsoLight or Reagent 5 for IsoSpark) at this time so that the run can be initiated immediately after cell stimulation and chip loading. Reagent A should be prepared, and all additional reagents should be setup in the instrument before proceeding. Refer to Chapter 4 for guidance on these steps.

Methods

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

- CRITICAL: CellTrace Far Red must be prepared fresh. Discard remaining stain—do not store.

  NOTE: DMSO tube is stored at -20°C after preparing the CellTrace Far Red Stock.

2. Prepare a working stock by diluting 2 µL of CellTrace Far Red into 998 µL of 1X PBS in a 1.5 mL Lo-Bind microcentrifuge tube (1:500 dilution). With the same pipette tip, pipette up and down 10 times to ensure all CellTrace Far Red has been released.
   a. With a P1000 set to 750 µL, gently pipette the stain working stock up and down **15 times**.
   b. **Gently vortex** the stain working stock for **5 seconds**.
   c. **Ensure working stock is mixed well.**

- 3. Prepare stain master mix by diluting 1 mL of CellTrace Far Red working stock into 9 mL of 1X PBS in a 15 mL centrifuge tube (1:10 dilution of working stock, 1:5,000 final dilution). With the same pipette tip, pipette up and down 10 times to ensure all CellTrace Far Red 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 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 flask or plate from with cells from incubator.

- 5. 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 plate.**

6. Add 1X PBS to flask or plate in the following volumes:

- For T75 Flask: 3 mL
- For T25 Flask: 2 mL
- For 6 Well Plate: 1 mL

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

7. Remove 1X PBS from flask or plate with a pipette.

8. Gently remix stain master mix.

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

9. Add stain master mix to each well or flask. **NOTE: For every 1 x 10^6 cells, 1 mL of well mixed stain master mix should be used. Ensure the stain master mix covers the bottom of the container.**

- For T75 Flask: 7-10 mL
- For T25 Flask: 3-5 mL
- For 6 Well Plate: 2 mL

10. Incubate for 15 minutes at 37°C in the dark.

11. Aspirate stain master mix from flask or well.

12. Add TrypLE Express Enzyme to the flask or plate in the following volumes:

- For T75 Flask: 5 mL
- For T25 Flask: 3 mL
- For 6 Well Plate: 1 mL

- **TIP: Gently rock flask or plate to ensure TrypLE is distributed evenly across entire surface area.**

13. Place flask or plate in incubator for 5 minutes.

14. After incubation, check under microscope to determine if cells are detached. If so, proceed to next step, if not place in incubator and continue to check every 90 seconds.

15. Rinse flask or plate with complete RPMI using the following volumes:

- For T75 Flask: 10 mL

- For T25 Flask: 6 mL
- For 6 Well Plate: 2 mL

- TIP: Dispense complete RPMI from top of plate/flask while angling the plate or flask so complete RPMI washes down all cells – while angled down, you can tilt flask side to side to ensure all cells are washed down with complete RPMI.

16. Transfer cell suspension to 15 mL centrifuge tube. Take 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.
17. If running stimulated and unstimulated cells, pipette half of the cell suspension into a 2nd 15 mL centrifuge tube.
18. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.

- CRITICAL: During this time, refer to Table 10 in Chapter 5 to determine volume of 1X PBS required to resuspend stimulated cell pellet.

19. After cells are centrifuged, check for cell pellet.
20. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to remove last bit of supernatant.
21. Using cell count from step 18, resuspend cell pellets in 1X PBS. Unstimulated cell suspension should be resuspended to a density of $1x10^6$ cells/mL. Stimulated cell suspension should be at volume determined based on Table 10 (Chapter 5).

Chapter 4: Chip and Reagent Thaw

Materials Required

IsoCode Chips in Vacuum Sealed Bag (-20°C)
50 mL tube containing reagent 2 for IsoLight or 50 mL tube containing reagent 5 for IsoSpark (-20°C)

Methods

- 1. Take 50mL tube containing reagent 2 for IsoLight or 50 mL tube containing reagent 5 for IsoSpark from -20°C to thaw at ambient temperature 90-120 minutes prior to use. CRITICAL: Ensure reagent 2 (IsoLight) or reagent 5 (IsoSpark) is completely thawed prior to Chip Loading (Chapter 6).
- 2. Take vacuum sealed bag containing IsoCode chips from -20°C. CRITICAL: Chips must stay sealed until Chip Loading (Chapter 6).

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

Chapter 5: Cell Stimulation

Materials Required

Sterile 1X PBS (Room Temperature)
2 x 70 µm Cell Strainer
4 x 15 mL Centrifuge Tubes (*Stimulated, Unstimulated)*
hEGF (-20°C)
hIFN-α (-20°C)
Calyculin A (-20°C)
Protease Phosphatase Inhibitor (PPI) Cocktail (4°C)
Cell Suspension(s) from Chapter 3

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

Methods

1. Place two 70 µm cell strainers on top of two new 15 mL centrifuge tubes.
2. Pre-wet each filter with 500 µL of 1X PBS by applying the tip of a P1000 directly on the filter.
3. Discard the two 15 mL centrifuge tubes containing 1X PBS.
4. Place each prepared filter on top of a new 15 mL centrifuge tube. NOTE: One tube will be used for unstimulated cell suspension and one for the stimulated cell suspension.

- CRITICAL: Protein phosphorylation is a highly transient process making stimulation time critical. Before adding any of the reagents mentioned in this section, be sure to have IsoCode chips thawed, reagents setup in the instrument and data upload location set.

5. Retrieve pre-made single use aliquots of hEGF, hIFN-α, and Calyculin A from -20°C.

- 6. Thaw aliquots at ambient temperature. Spin down in a mini centrifuge for 10 seconds to collect the contents at the bottom of the tubes. TIP: Ensure that contents are all in the bottom of the vial.

7. Prepare stimulant dilutions in 1X PBS. Using cell count from Chapter 3 step 18, determine the amount of each stimulant required for the stimulated cell suspension. See Table 9 for details on stimulant concentration. See Table 10 for example calculations.
    a. Dilute aliquot of hIFN-α 1:100. hIFN-α will be used at a 100X dilution in a 1:20 ratio (final concentration 50 ng/mL).
    b. Dilute aliquot of Calyculin A 1:10. Calyculin A will be used at a 10X dilution in a 1:50 ratio (final concentration 50 nM).
    c. hEGF will be used at stock concentration (no dilution necessary). hEGF will be used at stock concentration at a 1:20 ratio (final concentration 5 µg/mL)

Table 9: Concentration and Dilution Information for Stimulants and PPI

Stimulant or PPI	Stock Concentration	Dilution Factor	Concentration after Dilution	Final Concentration in Cells
hEGF	100 µg/mL	N/A	N/A	5 µg/mL
hIFN-α	100 µg/mL	1:100	1 µg/mL	50 ng/mL
Calyculin A	10 µM	1:10	1 µM	50 nM
PPI	100X	N/A	N/A	1X

8. Add protease phosphatase inhibitor cocktail to unstimulated cell suspension at a 1:100 ratio.
9. Add stimulants and protease phosphatase inhibitor cocktail to stimulated cell suspension. NOTE: Stimulated cell suspension will be at a final density of 1 x 10^6 cells/mL. For 100,000 cells, this would require a final volume of 100 µL.

Table 10: Examples for Stimulation and PPI Reagent Volumes for Different Cell Numbers

# of Cells	Vol. 1X PBS (µL)	Vol. Stock hEGF (µL)	Vol. 100X diluted hIFN-α (µL)	Vol. 10X diluted Calyculin A (µL)	Vol. PPI Cocktail (µL)	Total Volume (µL)
100,000	84	5	5	5	1	100
250,000	210	12.5	12.5	12.5	2.5	250
500,000	420	25	25	25	5	500

- 10. Pass each cell suspension through the preprepared strainer by applying the tip of a P1000 directly to the filter. NOTE: A strainer is used to separate cells that may have stuck together and ensures the cells are a single-cell suspension prior to loading. TIP: Apply the cell suspension to the same area the 1X PBS was applied.
- 11. Move immediately to Chapter 6. CRITICAL: Delaying loading of the chip will negatively impact results.

Chapter 6: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 4 Stained HeLa Cells at 1 x 10^6 cells/mL from Chapter 5

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 35 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 35 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 35 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 35 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 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 may be used in place of manual cell counting. Please pay close attention to any stains that have already been applied to cells as they may not be spectrally compatible with automated cell counting stains. If you are unsure, it is recommended to use manual cell counting.

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

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

   CRITICAL: If more than 200 cells/16 squares were counted, repeat count using a 1:5 or 1:10 dilution with 1X PBS or complete RPMI using a fresh sample aliquot.
5. Calculate the concentration of cells as follows:
   a. Concentration (cells/mL) = Average per square cell count x 10^4 x dilution factor
6. Calculate the number of cells as follows:
   a. Number of cells = Cell concentration (cells/mL) from D.1.5 x total volume of cell suspension (mL)
7. Calculate percent viable cells:
   a. % Viable cells = 100 x number of viable cells / [number of viable cells + number of dead cells]

Key: TIP, CRITICAL, OPTIONAL

Troubleshooting & References

Please contact Support at 844-476-7539 (toll free) or 475-221-8402 or email support@isoplexis.com with specific troubleshooting questions.

Problem	Possible Reason	Solution
Low quality cell count on chip *Cell Counting & Concentration related*	• Recommended cell concentrations not used • Issue with Cell Counting procedure • Trypan Blue may have debris • Trypan Blue is toxic	• Use recommended cell concentrations ensure cells are at $1x10^6$ cells/mL • Use appropriate dilutions recommended in Appendix D1 • Do a recount if initial count does not seem accurate • Quick spin Trypan Blue to pellet potential debris, remove aliquot from top of Trypan Blue. Start with fresh aliquot of Trypan Blue. • Thoroughly mix cells in well with pipette prior to transferring to tube • Count within 15 minutes of staining cells
Low quality cell count on chip *Stain Process related*	• Use of media other than the recommended media in protocol which could interact with cell stain • Use of stains not recommended in protocol • Recommended stain concentration, incubation time and/or incubation temperature not used • CellTrace Far Red was not stored at -20°C prior to use • Media not completely removed from cell pellet prior to staining	• Use complete RPMI media following recipe in Table 6 • Use Bruker validated stain • Follow staining steps as highlighted in Chapter 3 • Use only freshly prepared CellTrace Far Red per Chapter 3 • Ensure all media is removed from cells in step 3.6

Low quality cell count on chip *Technique Detail related*	• Bubbles loaded onto chip, especially at Chip Loading
	• Detection of potential artifacts such as debris, cell clumping, 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 step in 6.2 and 6.3 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 (35,000 cells per chip) (Chapter 6)
		• Use low protein binding centrifuge tubes

Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Stimulation step related*	• Recommended stimulation concentration was not used • Recommended stimulation duration was not used	• Use stimulation concentrations listed in Chapter 5 • Cells must be loaded immediately after addition of stimulants • Use recommended vendor as listed in Table 3

Key: ● TIP, ● CRITICAL, ● OPTIONAL