SAMPLE PREPARATION PROTOCOLS FOR PTMSCAN® ANALYSIS BY CELL SIGNALING TECHNOLOGY, INC.

CONTACT INFORMATION

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PTMSCAN® PROGRAM MANAGEMENT:

Jeffrey C. Silva, Ph.D.
Cell Signaling Technology, Inc.
3 Trask Lane, Room 2309
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978-826-6073; jsilva@cellsignal.com

PTMSCAN® PROFILING SERVICES WEBSITE:

http://www.cellsignal.com/services/index.html

PHOSPHOSITEPLUS®:

http://www.phosphosite.org/homeAction.do

Sample Submission Form for Project Reference # _____		
Sample #	**Species**	**Description**
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NOTES:		

Print multiple sheets if the total number of samples exceeds twenty-eight or recreate the form in Excel for electronic submission/preparation. Modify numbering of additional sheets accordingly.

SHIPPING INSTRUCTIONS FOR PTMSCAN® SAMPLES

All samples must be shipped to CST on dry ice by overnight courier.

NOTE: DO NOT ship any samples on a Thursday or Friday in order to prevent weekend holdover in transit and possible damage to valuable samples. It is preferable to ship materials on Monday or Tuesday.

Please notify by email the PTMScan® Project Manager at jsilva@cellsignal.com prior to overnight shipment. Include in the email communication, the overnight courier and tracking number to ensure efficient delivery. Please include in your email a description of the samples as they are labeled on the tubes. It is preferable that you label the tubes with numbers or letters and provide a hard copy word file with the full description for each sample number/letter. Also, please enclose a printed copy of the sample description with the overnight shipment. Please label the side of the box with your CST contract number. You will receive an email confirmation upon sample receipt.

NOTE: Please label the side of the box with your CST contract/project number (Q# or K#)

NOTE: See special instructions (next several pages, including declaration form) for shipping samples from outside the United States.

SHIP KINOMEVIEW™ & PTMSCAN® SAMPLES TO:

Jeffrey C. Silva
KinomeView™ & PTMScan® Project Manager
Cell Signaling Technology, Inc.
3 Trask Lane
Danvers, MA 01923
978-826-6073
jsilva@cellsignal.com

Procedure to send samples and other related material to CST from outside of the US

IMPORTANT NOTE: Please do not deviate from any aspect of the following shipping protocol. If you have any questions with the protocol, please contact the KinomeView™/PTMScan® Project Manager.

1.) Login to FedEx Ship Manager at fedex.com/Create a FedEx Ship Manager account and then log in.

(FedEx Ship Manager can be accessed by clicking the "Prepare Shipment" link under the Ship menu on FedEx.com)

2.) In the "To" field, fill out CST's address and phone number, along with the name of your Contact at CST.

Cell Signaling Technology Inc
3 Trask Ln
Danvers, MA 01923
#978-867-2300

3.) In the To: area, select:

Service Type: International Economy or International Economy Freight
Shipment Purpose: Sample
Total Customs Value: Appropriate value or $50 (if no value ascribed)
-along with all other starred fields.

IMPORTANT NOTE: Please DO NOT select “priority status” since this will not ensure proper handling of the materials through US customs.

4.) In the "Billing Details" field.

Bill transportation to: CST's FedEx account = #252799591
Bill duties/taxes/fees to: Recipient

5.) In Special Services, edit, and check off "Broker Select".

IMPORTANT NOTE: You will need to check off "Broker Select" to ensure proper handling of the materials through US customs.

6.) In the "Broker Select" field, add the following information:

Broker company name: CH Powell Company
Broker contact name: Peter F. Swett
Address 1: 440 William F McClellan Highway
City: East Boston
Postal Code: Ma 02128
Phone Number: 617-569-4400

7.) Click "Ship"

8.) In the "Commodity Summary" field, click "add new commodity", and then add the following information:

Commodity Description: "Rabbit Antibody", for example
Unit of Measure: each
Quantity: ?
Commodity Weight: ?
Customs Value: as entered on previous page.
Country of manufacture: your country.
Harmonized Code: 3002905150

9.) In the "Customs Documentation" field, click "Commercial Invoice".

10.) Click "Ship"

11.) Print the shipping label and commercial invoice, and include as paperwork with the shipment

12.) Please fill out the attached declaration form, and include as paperwork with the shipment.

13.) Package your sample appropriately for up to one week in transit. Attach the paperwork to your packaged sample and give the package to FedEx.

IMPORTANT NOTE: If the material must be stored cold, please make sure to write on the outside of the box "COLD STORAGE NECESSARY! -20C" or "REFRIGERATED STORAGE NECESSARY! 2C-8C". The -20 storage will be sufficient to extend the use of the packaged DRY ICE.

14.) E-mail the tracking number for your package to: your primary contact at CST (jsilva@cellsignal.com), distributors@cellsignal.com and PFSwett@chpowell.com).

DECLARATION FORM

PLEASE FILL OUT THIS FORM FOR ALL BIOLOGICAL SAMPLES GOING ABROAD

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1. SENDER:

2. ADDRESS AND TELEPHONE NUMBER:

3. FROM WHAT ANIMAL (IF ANY) DOES THE SAMPLE DERIVE:

4. WHAT IS THE SAMPLE STORED IN (I.E. SALINE SOLUTION / BOVINE SERUM). INCLUDE THE VOLUME AND TYPE OF CONTAINER:

5. FULL DESCRIPTION (AS MUCH INFORMATION AS POSSIBLE):

6. THE HISTORY OF THE PRODUCT:

7. WHAT WILL IT BE USED FOR:

8. COUNTRY OF ORIGIN:

RECIPIENTS NAME: Jeffrey C. Silva, Ph.D.

RECIPIENTS ADDRESS + TELEPHONE NUMBER:

Cell Signaling Technology, Inc.
3 Trask Lane
Danvers, MA 01923, USA

Telephone number: 978-826-6073

VALUE FOR CUSTOMS PURPOSES: US $1

These materials have no value to be declared and according to the U.S. Department of Agriculture do not require import permits or other documentation. Please refer to USDA/APHIS/Veterinary Services Guideline #1120/ Primate, Fish, Human and Rodent Cell Lines.

INDICATE TEMPERATURE FOR STORAGE: minus 20 degrees Celsius

NOTE: PLEASE GIVE AS MUCH INFORMATION AS POSSIBLE AS THIS WILL SPEED UP CLEARANCE OF YOUR SAMPLE.

SIGNATURE OF SENDER:_____

PRINTED NAME OF SENDER:_____

DATE: _____

PTMSCAN® UREA LYSIS BUFFER PREPARATION.

NOTE: After reviewing the protocols document, please schedule a teleconference with the KinomeView™/PTMScan® project manager to address any questions you may have with the protocol and finalize the details of your PTMScan® study (see contact information on page 2).

***** Urea Lysis Buffer must be prepared FRESH from stocks on the day of use. *****

PTMScan® Urea Lysis Buffer contains the following components:
20 mM HEPES (pH 8.0)
9.0 M urea
1 mM sodium orthovanadate (activated)
2.5 mM sodium pyrophosphate
1 mM ß-glycerol-phosphate.

NOTE: Prepare solutions with Milli-Q or equivalent purified water. DO NOT use any labware that has been in contact with detergents, such as Triton X-100, PEG, etc. These can interfere significantly with LC-MS/MS performance.

Make fresh 50 mL of Urea Lysis Buffer as follows:
5.0 mL of 200 mM HEPES, pH 8.0
27.0 gm urea**
0.5 mL of 100 mM sodium orthovanadate, *activated – see protocol below*
2.0 mL of sodium pyrophosphate (25X stock is 1.1 gm in 40 mL)
50 µL of ß-glycerol-phosphate (1000X stock is 2.2 gm in 10 mL)
Add water to 50 mL

NOTE: Urea must be Pierce Sequanal grade (catalog #29700). Other reagents may be purchased from Sigma or other high quality supplier.

NOTE: As urea dissolves in water, the solution cools down. A urea solution at 9 M is close to saturation. In order to bring all the solids into solution, stir the buffer in a beaker with warm (not hot) water.

Sodium Orthovanadate Activation (100 mM stock) for Urea Lysis Buffer involves depolymerization ("activation") by the following protocol.

***** Caution: The following steps should be performed in a fume hood *****

1. After dissolving sodium orthovanadate in water, adjust the pH to 10 using either 1 N NaOH or 1 N HCl, with stirring.
   The starting pH of the sodium orthovanadate may vary with different suppliers and lots (the chemical from Sigma typically has a starting pH of 12 and is adjusted to pH 10 with HCl. At pH 10, the solution will be yellow.
2. Boil the solution until it turns colorless and then let cool to room temperature.

3. Readjust the pH to 10 and repeat step 2 until the solution remains colorless and the pH is stable at 10. Adjust the final volume with water.
4. Store the activated sodium orthovanadate in 0.5 mL aliquots at -20°C. Thaw one aliquot for each experiment; use only once after thawing and do not re-freeze the thawed vial for future use. Discard if the solution turns yellow.

ADHERENT CELL PREPARATION FOR PTMSCAN® - ABBREVIATED PROTOCOL [RECOMMENDED].

**** Prepare Two Hundred Million (2 x 10^8) Adherent Cells ****

1. *Confirm cell viability and sub-confluence by microscopic inspection.*
2. *Proceed with the following steps one dish at a time.*
3. Dish #1: Pour off medium then pipette off remainder medium using a P-1000.
4. Wash gently and briefly the cells with 4°C PBS; pour off then pipette remainder of PBS.
5. Add 10 mL *freshly prepared* Urea Lysis Buffer to the dish.
6. Tilt dish side-to-side to distribute evenly and cover cells with Urea Lysis Buffer.
7. Dish #2: Pour then pipette off medium, wash with PBS then remove PBS immediately prior to transfer of Urea Lysis Buffer from Dish #1.
8. Use a disposable cell scrapper to scrape cells off of Dish #1 and transfer the lysate by pipetting to Dish #2. Scrape remaining buffer in Dish #1 into a 50 mL tube.
9. Repeat scrapping and transfer steps 6, 7 and 8 until all dishes of cells have been lysed and pooled in Urea Lysis Buffer.
10. Cap 50 mL screw-top tube, seal cap with Parafilm. Freeze on dry ice/ethanol for at least 30 minutes. Store overnight at -80°C if not shipping immediately.
11. Send frozen extracts on dry ice for OVERNIGHT courier (see Shipping Instructions on front page).

ADHERENT CELL PREPARATION FOR PTMSCAN® - DETAILED PROTOCOL.

**** Prepare Two Hundred Million (2 x 10^8) Adherent Cells ****

1. Grow 2 x 10^8 cells for each experimental condition. This will produce approximately 20-40 mg of total protein. This cell number corresponds to approximately ten 150 mm culture dishes grown to 70-80% confluency.

NOTE: Do not prepare cell lysates for PTMScan® with cells that are >80% confluent. Confirm cell viability and sub-confluence by microscopic inspection.

Proceed with the following steps one dish at a time until all dishes of cells have been harvested.

2. Dish #1: Pour off medium then remove remainder of medium with P-1000.

3. Gently add 10 mL of 4°C PBS, wash gently and briefly, then pour off and pipette off remainder of PBS.

4. Add 10 mL of Urea Lysis Buffer to Dish #1, scrape the cells off in the buffer and let the dish stand in tilted position after scraping the buffer.

NOTE: Urea Lysis Buffer will become viscous during cell lysis due to DNA.

5. Prepare Dish #2: remove medium, wash with PBS and remove PBS;

6. Pipette the Urea Lysis Buffer from Dish #1 to Dish #2. Then scrape the remainder of the lysate from Dish #1 (approximately 200 µL) into a round-bottom centrifuge tube.

7. Repeat the sequential washing, lysis and transfer of steps 5 and 6 for all the remaining dishes.

8. Pipette lysis buffer from last dish into the 50 mL tube described above. Scrape the remainder of lysis buffer from the last dish into the round-bottom centrifuge tube.

9. The cell lysate yield from 10 dishes will be 9–12 mL.

10. Sonicate the cell lysate. First cool on ice for 1 minute, then sonicate using a microtip set to 15 W output with 3 bursts of 30 seconds each. Cool on ice for 1 minute between each burst.

11. Centrifuge to clear the lysate. Centrifugation at 20,000 x g at 4°C for 15 minutes then transfer the SUPERNATANT (THIS IS YOUR SAMPLE) into a 50 mL screw-cap tube.

    Note: If cell lysate is left on ice for a prolonged period, the urea may precipitate. If a precipitate forms, remove from ice and warm slightly by hand until urea is in solution.

12. Cap the 50 mL sample tube. Seal the capped sample tube with Parafilm and place on dry ice/ethanol for at least 30 minutes, or longer if necessary for protein extract to freeze completely. If sample is not being shipped immediately, store at -80°C.

13. Send frozen protein extracts on dry ice by OVERNIGHT courier (see Shipping Instructions on front page).

SUSPENSION CELL PREPARATION FOR PTMSCAN® - ABBREVIATED PROTOCOL [RECOMMENDED].

**** Prepare Two Hundred Million (2 x 10^8) Adherent Cells ****

1. *Confirm cell viability and sub-confluence by cell staining and microscopic inspection.*
2. *Proceed with the following steps one dish at a time.*
3. Harvest cells by centrifugation at 200 x g (~1000 rpm, 16 cm radius) for 5 minutes at 4°C; pour and drain off medium.
4. Suspend gently and rinse cells with 20 mL of 4°C PBS.
5. Harvest cells by centrifugation as above. Pour and drain off PBS.
6. Add 10 mL of *freshly prepared* Urea Lysis Buffer (1 mL per 1.25 x 10^8 cells).
7. Transfer supernatant to screw cap tube, seal cap with Parafilm. Freeze on dry ice/ethanol for 30 minutes. Store overnight at -80°C if not shipping immediately.
8. Send frozen extracts on dry ice for OVERNIGHT courier (see Shipping Instructions on front page).

SUSPENSION CELL PREPARATION FOR PTMSCAN® - DETAILED PROTOCOL

**** Prepare Two Hundred Million (2 x 10^8) Adherent Cells ****

1. Grow 2 x 10^8 cells for each sample to be analyzed by PTMScan®; this will generate approximately 20-40 mg of total protein. Note: The volume of sub-saturated cell culture needed to yield 2 x 10^8 total cells will vary with cell type and should be determined empirically.

2. Transfer cell suspension to one or more centrifuge bottles, as necessary. Gently pellet cells by centrifugation at 200 x g (~1000 rpm, 16 cm radius) for 5 minutes at 4°C.

3. Carefully aspirate medium without disturbing the cell pellet.

4. Suspend gently the pelleted cells in 20 mL of 4°C PBS. Combine suspended cells if more than one bottle was used in step 2.

5. Gently pellet cells by centrifugation at 200 x g (~1000 rpm, 16 cm radius) for 5 minutes at 4°C.

6. Carefully aspirate PBS without disturbing the cell pellet.

7. Add 10 mL of Urea Lysis Buffer to the cell pellet and pipet to suspend.

NOTE: The lysate will be very viscous.

8. Sonicate the cell lysate. First cool on ice for 1 minute, then sonicate using a microtip set to 15 W output with 3 bursts of 30 seconds each. Cool on ice for 1 minute between each burst.

9. Centrifuge to clear the lysate. Transfer to a centrifuge tube and centrifugation at 20,000 x g at 4°C for 15 minutes then transfer the SUPERNATANT (THIS IS YOUR SAMPLE) into a 50 mL screw-cap tube.

10. Cap the 50 mL sample tube. Seal the capped sample tube with Parafilm and place on dry ice/ethanol for at least 30 minutes, or longer if necessary for protein extract to freeze completely. If sample is not being shipped immediately, store at -80°C.

11. Send frozen protein extracts on dry ice by OVERNIGHT courier (see Shipping Instructions on front page).

TISSUE/TUMOR/XENOGRAFT SAMPLES FOR PTMSCAN® - FLASH FREEZING PROTOCOL [RECOMMENDED].

**** Prepare five hundred milligrams (500 mg) of tissue for each PTMScan® analysis ****

NOTE: Where possible, such as with animal model and xenograft tissue samples, tissue representing each experimental condition should be pooled from at least three (3) different animals or xenograft tumors. This is to average biological variability for each condition.

NOTE: Xenograft tumors should no greater than one hundred fifty milligrams (150 mg) each, so as to ensure the health, unrelated to the treatment, of the xenograft is maintained.

1. Before harvesting any tissues, label cryo-vials appropriately for each sample. Label a sufficient number of cryo-vials for the PTMScan® samples plus an additional cryo-vial for reserved tissue.

NOTE: DO NOT use flip-top microfuge tubes.

2. Fill a Dewar flask with liquid nitrogen.

3. Harvest tissue. Separate approximately fifty milligrams (50 mg) of tissue for each experimental condition and reserve for other analyses, e.g. Western, IHC, etc. Place tissue in labeled cryo-vials and flash freeze in liquid nitrogen. Cover the Dewar and let the sample sit in liquid nitrogen for at least 5 min, or until completely frozen. If samples are not being shipped immediately, store samples at -80°C.

4. For shipping samples, transfer cryo-vials to Styrofoam cooler with dry ice and ship by OVERNIGHT courier (see Shipping Instructions on front page).

TISSUE/TUMOR/XENOGRAFT SAMPLES FOR PTMSCAN® - LYSATE PREPARATION PROTOCOL.

**** Prepare five hundred milligrams (500 mg) of tissue for each PTMScan® analysis ****

NOTE: Where possible, such as with animal model and xenograft tissue samples, tissue representing each experimental condition should be pooled from at least three (3) different animals or xenograft tumors. This is to average biological variability for each condition.

NOTE: Xenograft tumors should no greater than one hundred fifty milligrams (150 mg) each, so as to ensure the health, unrelated to the treatment, of the xenograft is maintained.

1. Harvest tissue. Separate approximately fifty milligrams (50 mg) of tissue for each experimental condition and reserve for other analyses, e.g. Western, IHC, etc. Flash freeze 50 mg samples in labeled cryo-vials by immersion in liquid nitrogen and store at -80°C.

2. Cut into small pieces the remaining 450 mg of each tissue sample and place in a round-bottom centrifuge tube.

3. Add 1 mL of *freshly prepared* Urea Lysis Buffer for each 100 mg of tissue.

4. Homogenize the samples using a Polytron set at maximum speed: 2 x 20 second pulses. Chill on ice for 1 minute between each pulse

5. Sonicate the cell lysate. First cool on ice for 1 minute, then sonicate using a microtip set to 15 W output with 3 bursts of 30 seconds each. Cool on ice for 1 minute between each burst.

6. Centrifuge to clear the lysate. Centrifugation at 20,000 x g at 4°C for 15 minutes then Transfer the SUPERNATANT (THIS IS YOUR SAMPLE) to a 50 mL screw-cap tube.

NOTE: Reserve 100 µL of each sample in a microfuge tube for protein concentration determination and Western analysis.

NOTE: If cell lysate is left on ice for a prolonged period, the urea may precipitate. If a precipitate forms, remove from ice and warm slightly by hand until urea is in solution.

7. Cap the 50 mL sample tube. Seal the capped sample tube with Parafilm and place on dry ice/ethanol for approximately 30 min, or until protein extract is completely frozen. If not shipping immediately, store at -80°C.

8. Ship samples frozen on dry ice by OVERNIGHT courier (see Shipping Instructions on front page).

SILAC DMEM PREPARATION & HEAVY-ISOTOPE LABELING IN CELL CULTURE.

NOTE: SILAC medium must contain no leucine, lysine or arginine.

NOTE: Prepare solutions with Milli-Q or equivalent purified water.

For one liter (1 L) LIGHT or HEAVY DMEM:

1. Use prepared "drop-out" DMEM medium (Sigma-Aldrich Cat. # D9443-500ml).
2. Add 21 mL of 5 mg/mL Leucine (Sigma L-8912).
3. Add 5 mL of penicillin (10,000 U/mL) and 5 ml of streptomycin (10 mg/mL).
4. Add Lysine and Arginine:
   - LIGHT DMEM: Add 50 mg normal-isotope Lysine (Sigma L-8662) and 50 mg normal-isotope Arginine (Sigma A-6969)
   - HEAVY DMEM: Add 52 mg heavy-isotope Lysine (U-^{13}C6, Cambridge Isotope Laboratories CLM-2247-0.25) and 50 mg heavy-isotope Arginine (U-^{13}C6, Cambridge Isotope Laboratories CLM-2265-0.25).
5. *[Add dialyzed serum as appropriate for cell culture.]*
6. Add water to a final volume of 1 L.
7. Sterilize media by filtration through 0.22 micron filter.

NOTE: Cells must be propagated through at least SIX to SEVEN (6 – 7) population doublings.

This is approximately equivalent to a 64-fold increase in cell number, which will ensure complete incorporation of ^{13}C-Lys and ^{13}C-Arg into cellular proteins. Splitting cells 1:3, 1:2.5 or 1:2 will require 5, 6, or 7 passages, respectively.

NOTE: Use 25 mL of media per 15 cm dish to ensure that cells are not limited in their growth and proliferation.

NOTE: The arginine and lysine are U $^{13}C_6$ for a + 6.0201 mass difference per amino acid incorporation.

NOTE: For RPMI use Sigma-Aldrich Cat. # R1780-500ml.