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

CONTACT INFORMATION

PTMSCAN® PROGRAM MANAGEMENT:

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PTMSCAN® PROTEOMICS WEBSITE:

http://www.cellsignal.com/proteomics

PHOSPHOSITEPLUS®:

http://www.phosphosite.org

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 mstokes@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: There are special instructions for shipping samples from outside the United States. CST will assist you with both the necessary forms as well as scheduling the shipment.

SHIP KINOMEVIEW™ & PTMSCAN® SAMPLES TO:

Matthew P. Stokes, Ph.D.
KinomeView™ & PTMScan® Project Manager
Cell Signaling Technology, Inc.
3 Trask Lane
Danvers, MA 01923
978-867-2467
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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 g urea**
0.5 ml of 100 mM sodium orthovanadate, *activated – see protocol below*
2.0 ml of sodium pyrophosphate (25X stock is 1.1 g in 40 mL)
50 µl of ß-glycerol-phosphate (1000X stock is 2.2 g in 10 mL)
Add water to 50 ml

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®.

**** 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 50ml conical bottom 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 50ml conical bottom tube.

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

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 cell lysate to freeze completely. If sample is not being shipped immediately, store at -80°C.

11. Send frozen cell lysates on dry ice by OVERNIGHT courier (see Shipping Instructions on page 4).

SUSPENSION CELL PREPARATION FOR PTMSCAN®

~ Prepare Two Hundred Million (2 x 10^8) Suspension 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. Gently resuspend the pelleted cells in 20 ml of 4°C PBS. Combine resuspended cells if more than one bottle was used in step 2 in a 50ml conical bottom tube.

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. 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 cell pellet to freeze completely. If sample is not being shipped immediately, store at -80°C.

8. Send frozen protein extracts on dry ice by OVERNIGHT courier (see Shipping Instructions on page 4).

TISSUE/TUMOR/XENOGRAFT SAMPLES FOR PTMSCAN®

**** 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 be 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 blotting, immunohistochemistry, 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 minutes, or until completely frozen. If samples are not being shipped immediately, store samples at -80^{o}C.

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