

# PhosphoScan® Kit (P-Tyr-100)

✓ 5 Assays



Cell Signaling  
TECHNOLOGY®

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This product is for *in vitro* research use only and is not intended for use in humans or animals.

Products Included	Kit Quantity
10X Lysis Buffer Mix	20 ml
Urea**	60 g
Vanadate (100 mM, 100X)	5 Vials
DTT (1.25 M)	5 Vials
Trypsin-TPCK Solution (1 mg/ml, 100X)	5 Vials
10X HEPES Stock (200 mM, pH 8)	30 ml
Sep-Pak® C <sub>18</sub> Columns (0.7 ml each)**	5 Units
Phosphotyrosine Mouse mAb (P-Tyr-100) Beads*	5 x 40 µl
10X IAP Buffer	10 ml
Control Peptides	1 Assay
ZipTips®**	5 Units

\*Product is specific to this kit

\*\*Stored at room temperature, packaged separately.

**Introduction:** PhosphoScan® Kit (P-Tyr-100) from Cell Signaling Technology (CST) allows for the purification and identification of tyrosine phosphorylation sites in cellular proteins when coupled with LC tandem mass spectrometry using CST's patent-pending PhosphoScan® technology. The assay is based on the specific enrichment of phosphotyrosine-containing peptides using an antibody against phosphotyrosine. Cells are lysed in a urea-containing buffer, and cellular proteins are digested by protease and fractionated by reversed-phase solid-phase extraction. Peptides are then subjected to immunoaffinity purification using Phosphotyrosine Mouse mAb (P-Tyr-100) #9411 coupled to protein G agarose beads\*. Overnight incubation ensures high-affinity binding of phosphotyrosine-containing peptides to P-Tyr-100 beads. Unbound peptides are removed through washing, and phosphotyrosine-containing peptides are eluted with dilute acid. Reversed-phase chromatography is performed on

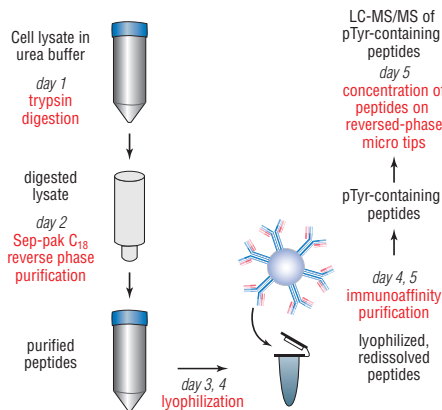
microtips to separate phosphopeptides from antibody and to concentrate them for LC tandem mass spectrometry.

The kit also contains control peptides from 2x10<sup>6</sup> Jurkat cells that were treated with pervanadate and calyculin A. These control peptides allow the investigator to determine the efficiency of the immunoaffinity purification (IAP) step and the LC-MS/MS analysis (1 assay).

**Background:** Tyrosine phosphorylation plays a key role in cellular signaling (1). In cancer, unregulated tyrosine kinase activity can drive malignancy and tumor formation by generating inappropriate proliferation and survival signals (2). Current phosphoproteomic approaches generally reveal only small numbers of tyrosine phosphorylation sites, in keeping with the low level of phosphotyrosine relative to phosphoserine and phosphothreonine residues (3). PhosphoScan® Kit, based on CST's published method (4,5), isolates large numbers of cellular phosphotyrosine-containing peptides. This allows the investigator to obtain a global overview of tyrosine phosphorylation in the cell, covering many classes of proteins without preconceived biases about where tyrosine phosphorylation sites will be found.

#### Background References:

- (1) Schlessinger J. (2000) *Cell* 103, 211–225.
- (2) Blume-Jensen, P. & Hunter, T. (2001) *Nature* 411, 355–365.
- (3) Mann, M. et al. (2001) *Trends Biotechnol.* 20, 261–268.
- (4) Rush, J. et al. (2005) *Nat. Biotechnol.* 23, 94–101.
- (5) Rush, J. et al. (2003) U.S. Patent Publication 20030044848.



#### Storage:

Upon first use, please store the items at the following temperature conditions:

1. Phosphotyrosine Mouse mAb (P-Tyr-100) beads, 100X vanadate, DTT, control peptides, trypsin-TPCK solution: -20°C.
2. 10X lysis buffer mix\*\*\*, 10X HEPES Stock\*\*\*: 2-8°C.
3. Sep-Pak® C<sub>18</sub> columns, ZipTips®, urea, 10X IAP buffer\*\*\*: Room temperature.

\*\*\*These components will precipitate out of solution at their shipping temperature, -20°C. Warm at 25-30°C and mix until dissolved. Store 10X lysis buffer mix and 10X HEPES stock at 2-8°C. Dilute 10X IAP buffer 10-fold. Store diluted IAP buffer (1X) at 2-8°C.

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(i) **Notice to Non-Profit/Academic Purchasers:** The purchase of this kit product includes the limited right and license to use the kit in the practice of any technology or methods described and claimed in U.S. Patent Publication 7,198,896 and foreign equivalents, "Immunoaffinity Isolation of Modified Peptides from Complex Mixtures", Rush et al., Pub. Date March 6, 2003 (and equivalents), provided such kit is not used in research or development projects funded in whole or in part by For-Profit/Corporate sponsors or in high-throughput screening projects resulting in information that will be offered to For-Profit/Corporate entities on a fee-for-access basis (which use(s) of purchased kit product, in any field, require a separate commercial use license from Cell Signaling Technology, Inc. ).

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For license inquiries, please contact Chris Bunker, Ph.D., Director of New Business Development, Cell Signaling Technology, Inc. (Ph: 978-867-2307, email: cbunker@cellsignal.com)

The kit contains control peptides that may be employed to check the efficiency of the Immunoaffinity Purification (IAP) step and the LC-MS/MS analysis. These control peptides are from 2x10<sup>8</sup> Jurkat cells that were treated with pervanadate and calyculin A (for one IAP assay).

After IAP and concentration on reversed-phase micro tips, a majority of the following 40 peptides should be seen as high scoring peptides in the LC-MS/MS analysis of the Jurkat/pervanadate/calyculin A control. Peptide homologs resulting from incomplete or more complete digestion may also be observed, as well as lesser or higher phosphorylated forms of the peptides. A very good analysis on an LCQ tandem mass spectrometer will result in 100 or more high scoring unique peptide sequences.

	Phospho-peptide	Protein	Phospho-site
1	LIEDNEY*TAR	LCK	Y394
2	LNK*KPPPQK	Rho GDI 2	Y24
3	SDY*DGIGSRGDR	DEAD/H box polypeptide 3	Y104
4	GRSDY*DGIGSR	DEAD/H box polypeptide 3	Y104
5	RIDTLNSDGY*TPEPAR	ZAP-70	Y292
6	IDTLNSDGY*TPEPAR	ZAP-70	Y292
7	ALGADDSY*Y*TAR	ZAP-70	Y492, 493
8	ALGADDSY*Y*TAR	ZAP-70	Y493
9	ERPPPVPNPDY*EPIRK	CD3E antigen	Y188
10	DLY*SGLNQR	CD3E antigen	Y199
11	SADAPAY*QQGQNQLY*NELNLGR	CD3ζ	Y64, 72
12	SADAPAY*QQGQNQLY*NELNLGR	CD3ζ	Y72
13	GFGSFRFPSGNQGGAGPSQGSQGG TGGSVYTENDDL*G	valosin-containing protein	Y805
14	GFPSGNQGGAGPSQGSQGGTGGSV Y*TEDNDDDL*G	valosin-containing protein	Y796, 805
15	GEPNVS*ICSR	GSK-3α	Y279
16	VKEEGY*ELPYNPATDDY*AVPPPR	p62dok1	Y398, 409
17	SHNSALY*SQVQK	p62dok1	Y449
18	IGEGTY*GVVYK	cdc2	Y15
19	LCDFGSASHVADNDITPY*LVSR	ser/thr protein kinase PRP4K	Y849
20	NEENIY*SVPHDSTQGK	glucocorticoid receptor DNA binding factor 1 iso. 1a	Y1105
21	HTDDEMTGY*VATR	p38α MAPK	Y182
22	KTPQGPPEIY*SNTQFPPLQSTAK	similar to hypothetical protein H41	Y107
23	IKEEMSKDQPDY*AMY*SR	dedicator of cytokinesis 2	Y209
24	FQDVGPQAPVGSVY*QK	HIP-55	Y162
25	SSTETCY*SAIPK	filamin B, β	Y2502
26	STLQDSDEY*SNPAPLPLDQHSR	leupaxin	Y22
27	NTDY*TELHQQNTDLIY*QTGPK	golgin subfamily a, 5	Y42, 54
28	TEDSIRDY*EDGMEVDTTPTVAGQ FEDADVDH	chloride channel, nucleotide-sensitive, 1A	Y214
29	NAEDCLY*ELPENIR	hepatocell. carcinoma-assoc. antigen 59	Y72
30	EKAEERPTFDYLQSVLDDFYTATEGQY*QQQP	Lyn kinase	Y508
31	AERPTFDYLQSVLDDFYTATEGQY*QQQP	Lyn kinase	Y508
32	VITNQYNNPAGLY*SSENISNFNNALESK	enigma homolog	Y151
33	VITNQY*NNPAGLYSSENISNFNNALESK	enigma homolog	Y144
34	VITNQY*NNPAGLY*SSENISNFNNALESK	enigma homolog	Y144, 151
35	ADGY*EPPVQESV	40S ribosomal protein S3a	Y356
36	KDPDERPTFEYIQSFLEDYFTATEPQY*QPGENL	Yes kinase	Y537
37	KDPEERPTFEYIQSFLEDYFTATEPQY*QPGENL	Fyn kinase	Y528
38	KEPEERPTFEYLQAFLEDYFTSTEPQY*QPGENL	Src kinase	Y530
39	LVNEAPVY*SVY*SK	signal transducing adaptor molecule 2	Y371, 374
40	NGSEADIDEGLY*SR	ubiquitin-activating enzyme E1	Y55

## Solutions and Reagents

<b>Cell lysis</b>	<ul style="list-style-type: none"> <li>• 10X Lysis Buffer*: 20 mM HEPES, 25 mM sodium pyrophosphate, 10 mM <math>\beta</math>-glycerophosphate (20 ml)</li> <li>• Urea (60 g solid; add to lysis buffer mix at 9 M)</li> <li>• Vanadate (100X stock; 5 vials)</li> </ul>
<b>Carboxamidomethylation and trypsin-TPCK digestion</b>	<ul style="list-style-type: none"> <li>• 10X HEPES Stock*</li> <li>• 1.25 M DTT (5 vials)</li> <li>• trypsin-TPCK solution (100X, 1 mg/ml)</li> </ul>
<b>Peptide purification</b>	<ul style="list-style-type: none"> <li>• Sep-Pak<sup>®</sup> C<sub>18</sub> columns, volume 0.7 ml (5 units)</li> </ul>
<b>Immunoaffinity purification (IAP)</b>	<ul style="list-style-type: none"> <li>• 5 x 40 <math>\mu</math>l Phosphotyrosine Mouse mAb (P-Tyr-100) beads (80 <math>\mu</math>l slurry in IAP buffer containing 50% glycerol)</li> <li>• 10X IAP buffer* (10 ml)</li> <li>• Control peptides from 2 x 10<sup>8</sup> Jurkat cells treated with pervanadate/calculinA (one assay)</li> </ul>
<b>Concentration and purification of peptides for LC-MS</b>	<ul style="list-style-type: none"> <li>• ZipTips<sup>®</sup> (5 Units)</li> </ul>

\*These components will precipitate out of solution at their shipping temperature, -20°C. Warm at 25-30°C and mix until dissolved. Store 10X lysis buffer mix and 10X HEPES stock at 2-8°C. Dilute 10X IAP buffer 10-fold. Store diluted IAP buffer (1X) at 2-8°C.

**Not provided:** Trifluoroacetic acid, Acetonitrile and Iodoacetamide.

## Cell Lysis and Protein Digestion

### Abridged Protocol

#### A. Preparation of cell lysate, suspension cells

- A1. Use 2 x 10<sup>8</sup> cells/experiment (20-40 mg protein).
- A2. Centrifuge cells, carefully remove supernatant and at room temperature add 2 ml lysis buffer (contains 9 M urea) to the cell pellet. Lyse by pipetting up and down and sonicating, and clear the lysate by centrifugation.

#### B. Preparation of cell lysate, adherent cells

- B1. Use 2 x 10<sup>8</sup> cells/experiment (20-40 mg protein). This cell number approximately corresponds to ten 150 mm culture dishes grown to 70-80% confluency.
- B2. After removal of medium, scrape cells in 10 ml lysis buffer/10 plates. Sonicate and clear the lysate by centrifugation.

#### C. Carboxamidomethylation of proteins

- C1. Add 1/10 vol. of 45 mM DTT to the cleared cell supernatant, mix well and incubate at 60°C for 20 minutes.

- C2. Cool the solution in ice/water to room temperature.

- C3. Add 100 mM iodoacetamide at an equal volume as the DTT solution, mix well, incubate for 15 minutes at room temperature in the dark.

#### D. Trypsin digestion

- D1. Dilute 4-fold to a final concentration of 2 M urea, 20 mM HEPES buffer, pH 8.0. Mix.
- D2. After dilution and mixing, remove a small aliquot, e.g. 50  $\mu$ l, for SDS-PAGE analysis.
- D3. Add 1/100 volume of a 1 mg/ml trypsin-TPCK solution and digest overnight at room temperature.
- D4. Analyze the lysate before and after digest on an 11-12% polyacrylamide gel to check for complete digestion.

### Detailed Protocol

#### A. Solutions and Reagents

*Note: Prepare solutions with Milli-Q or equivalently purified water*

- A1 Lysis Buffer: 20 mM HEPES pH 8.0, 9 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate.

10X lysis buffer mix will precipitate out of solution at -20°C. Warm at 25-30° and mix until dissolved. Store at 2-8°C.

For 20 ml solution, add 2 ml of 10X lysis buffer mix, 10.8 g urea and 0.2 ml of 100 mM sodium vanadate. This buffer must be prepared freshly for each experiment.

- A2. DTT solution: Dilute 180  $\mu$ l stock into a final volume of 5 ml with water for a 45 mM solution, freshly prepared for each experiment.

- A3. Iodoacetamide solution, 100 mM: Dissolve 190 mg iodoacetamide in water to a final volume of 10 ml. After weighing the powder, store in the dark and add water only immediately before use.

## Cell Lysis and Protein Digestion (continued)

### B. Preparation of cell lysate, suspension cells

B1. Grow  $2 \times 10^8$  cells/experiment (20-40 mg protein).

*Note: Cells do not need to be washed before lysis even if they are harvested after growth in serum-containing medium. If the medium is removed carefully from the cell pellet, residual serum protein will not interfere with the analysis.*

B2. Centrifuge cells, carefully remove supernatant and at room temperature add 2 ml lysis buffer mix to the cell pellet. Pipet the slurry up and down a few times, and cool on ice before sonication.

B3. Using a microtip, sonicate at 10 W output with 3 bursts of 30 seconds each. Cool on ice for 1 minute between each burst. Clear the lysate by centrifugation at  $20,000 \times g$  for 15 minutes and collect the supernatant into a new tube.

*Note: Do not freeze the cleared cell supernatant, proceed immediately to protease digest. Upon freeze/thaw protein loss by precipitation may occur.*

### C. Preparation of cell lysate, adherent cells

C1. Grow  $2 \times 10^8$  cells/experiment (20-40 mg protein). This cell number approximately corresponds to ten 150 mm culture dishes grown to 70-80% confluency.

*Note: Cells do not need to be washed before lysis if they are harvested after serum starvation in culture medium. If they are harvested after growth in serum-containing medium, a quick wash with 10 ml of PBS per plate, just before adding lysis buffer, is recommended. In both cases, fluid should be removed carefully from the plates before lysis.*

C2. Take a set of five 150 mm culture dishes, remove the medium from the first one by decanting, let stand in sharply tilted position for 30 seconds so that the rest of the medium flows to the bottom edge; take off the remainder of the medium at the bottom edge with a 1 ml micropipettor.

C3. Add 10 ml of lysis buffer to the culture dish, scrape the cells off in the buffer, let the dish stand in tilted position after scraping the buffer to the bottom edge of the tilted dish. Remove the medium from the next dish as above. Pipet from the lysis buffer as much as reasonably possible in a short time from the first dish to the next one while leaving the first dish tilted with the lid on, scrape cells from the second dish and so on – until the cells from all the 5 dishes have been scraped into the lysis buffer.

*(During lysis, the buffer becomes more and more viscous, until it is like a gel, due to DNA from the cells.)*

C4. Remove the remainder of lysis buffer from all dishes except the last one: scrape the buffer onto the bottom edge of the tilted dishes, aspirate with a micropipettor (0.5 – 1 ml volume yield) and combine with the last dish containing most of the lysis buffer.

C5. Using another set of 5 dishes, treat as above; use the extraction buffer from the first set of 5 dishes (aspirate lysis buffer from the last dish of this set with a 10 ml pipet).

C6. Scrape all lysis buffer from all dishes onto the last one (containing the most buffer), aspirate the lysis buffer with a 10 ml pipet into a 15 ml tube. Scrape the remainder of lysis buffer from the tilted last dish and combine with the rest of buffer in the tube.

C7. From 10 dishes, the yield will be 9–12 ml lysate.

C8. Cool on ice before sonication. Using a microtip, sonicate at 15 W output with 3 bursts of 30 seconds each. Cool on ice for 1 minute between each burst. Clear the lysate by centrifugation at  $20,000 \times g$  for 15 minutes and collect the supernatant into a new 50 ml tube.

*Note: Do not freeze the cleared cell supernatant, proceed immediately to protease digest. Upon freeze/thaw protein loss by precipitation may occur.*

### D. Carboxamidomethylation of proteins (inactivation of enzymatic activity)

D1. Add 1/10 volume of 45 mM DTT to the cleared cell supernatant (e.g. 1 ml if the supernatant is 10 ml), mix well, place the tube into a beaker containing water that is warmed up to 60°C and place the beaker into a 60°C oven for 20 minutes.

D2. Cool the solution in ice/water for approximately 10 minutes until it has reached room temperature (tube should feel neither warm nor cold).

D3. Add 100 mM iodoacetamide at an equal volume as the DTT solution, mix well, incubate for 15 minutes at room temperature in the dark.

### E. Trypsin digestion

E1. Dilute 4-fold to a final concentration of 2 M urea, 20 mM HEPES buffer, pH 8.0.

e.g. For 10 ml lysate supernatant:

10 ml lysate supernatant  
2 ml DTT/iodoacetamide  
3 ml 200 mM HEPES pH 8.0 (10X stock) \*  
25 ml water

Final volume: 40 ml

\*only 3 ml is added here because the 10 ml of lysate supernatant was previously buffered with 20 mM HEPES.

10X HEPES stock will precipitate out of solution at -20°C. Warm at 25-30°C and mix until dissolved. Store at 2-8°C.

E2. After dilution and mixing, remove a small aliquot, e.g. 50  $\mu$ l, for analysis on SDS-PAGE.

E3. Add 1/100 volume of the 1mg/ml trypsin-TPCK solution and digest overnight at room temperature.

E4. Analyze the lysate before and after digest on an 11-12% polyacrylamide gel to check for complete digestion.

## Sep-Pak® C<sub>18</sub> Purification of Lysate Peptides

### Abridged Protocol

#### A. Acidification of digested cell lysate

A1. Add 1/20 volume of 20 % TFA to the digest for a final concentration of 1 % TFA, let stand for 10 minutes.

A2. Centrifuge the acidified peptide solution for 5 minutes at 2,000 x g to remove the precipitate, decant peptide-containing supernatant into new tube.

#### B. Peptide Purification

B1. Pre-wet the 0.7 ml Sep-Pak® C<sub>18</sub> column (supplied) with 5 ml 100% acetonitrile (MeCN).

B2. Wash with 7 ml 0.1 % trifluoroacetic acid (TFA).

B3. Load acidified and cleared digest (see above).

B4. Wash column with 12 ml 0.1 % TFA.

B5. Wash column with 2.8 ml of 5 % MeCN, 0.1 % TFA.

B6. Elute peptide step-wise with 1.4 ml each of 10, 15, 20, 25, 35, 40 % MeCN in 0.1 % TFA into a 15 or 50 ml polypropylene tube.

B7. Lyophilize frozen peptide solution.

### Detailed Protocol

The purification is performed at room temperature on 0.7 ml columns from Waters, cat # WAT051910 (supplied in kit).

#### Background:

Sep-Pak® C<sub>18</sub> purification entails reversed-phase (hydrophobic) solid-phase extraction with size exclusion (MW 20,000). Peptides and lipids bind to the chromatographic material. Large molecules such as DNA, RNA and most protein, as well as hydrophilic molecules such as many small metabolites, do not. Peptides are eluted from the column by increasing acetonitrile (MeCN) concentrations up to 40 % (vol/vol) and separated from lipids, which elute around 60% MeCN and above.

#### Comments:

Peptides from up to 2 x 10<sup>9</sup> cells (about 20-40 mg peptide) can be purified on one column.

Lysate peptides should be purified as soon as possible after digest. If the purification cannot be performed the same day that the digest is finished, keep the digest for 1 to 3 days in a refrigerator, preferably at 0°C, i.e. in a bucket filled with ice and a lid on top. Do not freeze; upon thawing this may lead to peptide loss by precipitation.

#### A. Solutions and Reagents

*Note: Prepare solutions with Milli-Q or equivalently purified water. Organic solvents (trifluoroacetic acid, acetonitrile) should be of the highest grade. All percentage specifications for solutions are vol/vol.*

A1. 20 % trifluoroacetic acid (TFA):  
add 10 ml TFA to water to a total volume of 50 ml.

A2. solvent A (0.1 % TFA):  
add 5 ml 20 % TFA to 995 ml water.

A3. solvent B (0.1 % TFA, 40 % acetonitrile):  
add 400 ml acetonitrile (MeCN) and 5 ml 20 % TFA to 500 ml water, fill to 1 liter.

A4. solutions for peptide elution:		
make 40 ml of:	solvent A	solvent B
5 % MeCN, 0.1 % TFA	35ml	5 ml
10 % MeCN, 0.1 % TFA	30 ml	10 ml
15 % MeCN, 0.1 % TFA	25 ml	15 ml
20 % MeCN, 0.1 % TFA	20 ml	20 ml
25 % MeCN, 0.1 % TFA	15 ml	25 ml
30 % MeCN, 0.1 % TFA	10 ml	30 ml
35 % MeCN, 0.1 % TFA	5 ml	35 ml

#### B. Acidification of digested cell lysate

*Note: Before loading the peptides from the protein digest on the column, the digest must be acidified with TFA for efficient peptide binding.*

B1. Add 1/20 volume of 20 % TFA to the digest for a final concentration of 1 % TFA, let stand for 10 minutes.

B2. Centrifuge the acidified peptide solution for 5 minutes at 2,000 x g to remove the precipitate, decant peptide-containing supernatant into new tube.

#### C. Peptide Purification

*Note: Application of all solutions is by gravity.*

C1. Connect a 10 cc reservoir (10 cc syringe with plunger removed) to the SHORTER END of the column.

C2. Pre-wet the column with 5 ml 100 % MeCN.

C3. Wash with 7 ml (10 vol., applied as 2 x 3.5 ml) solvent A.

C4. Load acidified and cleared digest (see above).

C5. Wash column with 12 ml (applied as 1 + 5 + 6 ml) of solvent A.

C6. Wash column with 2.8 ml (4 vol., applied as 2 x 1.4 ml) of 5% MeCN, 0.1% TFA.

C7. Elute peptide step-wise with 1.4 ml each of 10, 15, 20, 25, 35, 40% MeCN in 0.1% TFA into a 15 or 50 ml polypropylene tube.

C8. Lyophilize frozen peptide solution for two days.

#### Notes:

1. Each time solution is applied to the column (except upon pre-wetting with pure MeCN), air bubbles form on the narrow inlet of the column. These can be removed with a gel-loader tip placed on a 200 µl micropipettor.

2. The lysate digest may have a much higher volume than the 10 cc reservoir (up to 50-60 ml from adherent cells) and therefore must be applied in several fractions. Theoretically, applying the peptide over a 60 cc reservoir is much simpler. However, under these circumstances the flow rate of the column slows dramatically, and air bubbles (see above) tend to form over time at the inlet of the column and must be removed periodically.

## Sep-Pak<sup>®</sup> C<sub>18</sub> Purification of Lysate Peptides (continued)

3. It is advisable to wash the 10 cc reservoir after sample loading: uncouple from column, rinse 3 times with distilled water, re-connect.

4. In case flow rates slow down dramatically upon/after loading of sample (which happens rarely if the method is followed as described), the purification procedure can be accelerated by gently applying pressure to the column using the plunger that was originally removed (again make sure to remove air bubbles from the narrow inlet of the column before doing so). Do not apply vacuum.

5. Elution is performed in small steps of increasing MeCN concentration, instead of a one-step elution with a 40% MeCN solution. The reason is the complex molecular behaviour of peptides. At lower MeCN concentrations more hydrophilic parts of the peptide may interact with the beads, at higher MeCN concentrations more hydrophobic parts of the peptide may interact. As a result, the lowest interaction with the beads actually may occur at intermediate MeCN concentrations, where the sum of either type of interactions is at its minimum.

6. For lyophilization either quick-freeze in ethanol/dry ice or freeze in  $-80^{\circ}\text{C}$  freezer for 2 hours or overnight. Frozen peptide is stable at  $-80^{\circ}\text{C}$  for several weeks. After lyophilization, peptide is stable at  $-80^{\circ}\text{C}$  for several months (seal the tightly closed tube with parafilm for storage).

Before or after lyophilization the Phosphoscan<sup>®</sup> procedure can be interrupted. Once the lyophilized peptide is dissolved in IAP buffer (see next step), continue to the end of the procedure.

7. Lyophilization must be carried out for two days. In most cases the peptide is dry after one day, but residual traces of TFA may lower the pH upon reconstitution with buffer for the immunoaffinity purification.

## Immunoaffinity Purification (IAP)

### Abridged Protocol

1. Dissolve peptide in 1.4 ml IAP buffer.
2. Clear solution by centrifugation for 5 minutes at 2,000 x g.
3. Transfer the peptide solution into the microfuge tube containing phosphotyrosine antibody beads.
4. Incubate overnight on a rotator at 4°C.
5. Wash beads 3 times with 1 ml IAP buffer each, followed by 2 washes with 1 ml water each.
6. Add 55 µl of 0.15% TFA to the beads for elution, mix and let stand at room temperature for 10 minutes.
7. Mix and collect eluate by centrifugation, wash beads with 45 µl of 0.15% TFA and combine with first eluate.

### Detailed Protocol

#### Solutions and reagents

*Note: Prepare solutions with Milli-Q or equivalently purified water. Trifluoroacetic acid should be of the highest grade. All percentage specifications for solutions are vol/vol.*

#### Provided in kit:

10X IAP buffer.

IAP buffer (1X): 50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl. Dilute 10X IAP buffer 10-fold. Store diluted IAP buffer (1X) at 2-8°C.

#### Procedure

1. Briefly spin tube with lyophilized peptide to collect all peptide at the bottom.
2. Add 1.4 ml IAP buffer to the lyophilized peptide and let stand for 5 minutes. Peptides will start to dissolve.
3. Further dissolve peptides under gentle shaking for 30 minutes at room temperature, optionally aided by brief sonication in a sonicator waterbath.

*Note: After dissolving the peptide in IAP buffer, check the pH of the peptide solution by spotting less than 1 µl on pH indicator paper (or on the relevant segment of a multi-panel pH indicator strip). The pH should be close to neutral, or at least not lower than 5-6. In the rare cases that the pH is more acidic (due to insufficient removal of TFA from the peptide under suboptimal conditions of lyophilization) titrate the peptide solution with a concentrated Tris solution which has not been adjusted for pH. Do not use strong base.*

4. Clear solution by centrifugation for 5 minutes at 2,000 x g (the pellet of insoluble matter may at times seem considerable, but this will not pose a problem since most of the peptide will be dissolved nonetheless). Cool on ice.
5. Transfer the peptide solution into the microfuge tube containing phosphotyrosine antibody beads (40 µl; 80 µl slurry).
6. Incubate overnight on a rotator at 4°C.
7. Centrifuge at 1,500 x g for 1 minute and remove supernatant with a 1 ml micropipettor.

*Note: If the cells were directly harvested from culture medium without PBS washing, some of the Phenol Red pH indicator will remain (it co-extracts during the Sep-Pak® C<sub>18</sub> purification of peptides) and color the peptide solution yellow. This coloration has no effect on the immunoaffinity purification step.*

*Note: All subsequent wash steps are at 0-4°C.*

*Note: In all wash steps except the last one, the supernatant should be removed reasonably well, but it does not have to be done to the last few microliters.*

8. Add 1 ml of IAP buffer to the beads, mix by inverting tube 5 times, centrifuge at 1,500 x g for 1 minute and remove supernatant with a 1 ml micropipettor.
9. Repeat step 8 two times.
10. Add 1 ml water to the beads, mix by inverting tube 5 times, centrifuge at 1,500 x g for 1 minute and remove supernatant with a 1 ml micropipettor.
11. Add 1 ml water to the beads, mix by inverting tube 5 times, centrifuge at 1,500 x g for 1 minute and remove supernatant with a 1 ml micropipettor until about 100 – 200 µl is left.
12. Centrifuge at 1,500 x g for 5 seconds to remove fluid from the tube walls, and carefully remove the supernatant to completion with a 200 µl micropipettor (using a gel loading tip can aid in complete removal of the wash).
13. Add 55 µl of 0.15% TFA to the beads, tap the bottom of the tube several times (do not vortex) and let stand at room temperature for 10 minutes.

*Note: In this step, phosphopeptides will elute.*

14. Tap the bottom of the tube again, centrifuge at 1,500 x g for 1 minute, remove the supernatant and add it to another microcentrifuge tube.
15. Add 45 µl of 0.15% TFA, tap the bottom of the tube a few times, centrifuge at 1,500 x g for 1 minute and combine the supernatant with the first eluate, mix.

## Concentration and Purification of Peptides for LC-MS on ZipTip®

### Note:

Purification on Stop and Go extraction tips (StageTips) gives superior yield and results in about 10-20% more peptide identifications upon LC-MS/MS; however, for consistent results some experience with the method is required.

**Reference:** Rappsilber, J., Ishihama, Y. & Mann, M. (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* 75, 663–670.

### Abridged Protocol

1. Divide the eluate into 7 aliquots of 14  $\mu$ l each, pipetted into microfuge tubes.
2. Pre-wet the ZipTip® two times with 10  $\mu$ l each of 40 % MeCN, 0.1 % TFA.
3. Equilibrate the tip by washing four times with 10  $\mu$ l each of 0.1% TFA.
4. Bind the sample by pipetting each aliquot 10 times.
5. Wash the tip 4 times with 10  $\mu$ l each of 0.1% TFA.
6. Elute peptide with 1  $\mu$ l of 40% MeCN, 0.1% TFA by pipetting 4 times up and down, the last time into the sample vial.

### Detailed Protocol

#### A. Solutions and Reagents

A1. Solvent A and solvent B, see Sep-Pak® peptide purification protocol

*Note: Solvent B contains a high concentration of volatile organic solvent. Tubes containing small volumes of these solutions should be prepared immediately before use and should be kept capped as much as possible, because the organic components evaporate quickly.*

*Note: Prepare solutions with HPLC-grade water. Organic solvents (trifluoroacetic acid, acetonitrile) should be of the highest grade.*

*Note: If the tips are allowed to dry after they have been wetted, peptides will bind irreversibly to the sorbent. When dispensing solutions from the ZipTip®, do not force the micropipettor to the second, "hard" stop position, except at the last step, when pipetting the peptide solution into the sample vial for LC-MS. After the tip has been wetted, work quickly and without interruption.*

#### B. Procedure

B1. Centrifuge the tube containing the IAP eluate to collect at the bottom any small amount of agarose particles that may have been transferred from the beads used for IAP. Divide the eluate into 7 aliquots of 14  $\mu$ l each, pipetted into 1.5 ml microfuge tubes. Make sure that no agarose particles are carried over in the last aliquot. In uncertain cases, it is better to refrain from transferring the last few microliters of the IAP eluate.

*Note: The sample has to be pre-aliquotted because ZipTips® cannot be used for measuring volumes of sample aliquots: the tip's sorbent resists flow.*

B2. Attach a ZipTip® to a 20  $\mu$ l micropipettor set for 10  $\mu$ l. Wet the dry sorbent by aspirating solvent B twice. When expelling the solution, briefly touch the droplet of liquid at the end to a lint-free tissue.

B3. Equilibrate the tip by aspirating 10  $\mu$ l each of solvent A four times. Expel the solution onto a lint-free tissue.

B4. Place the tip into the first sample aliquot prepared in step 1. Turn the micropipettor's volume adjustment dial until the entire volume is drawn into the tip. Aspirate and expel the sample into the tip 10 times to bind peptide to the sorbent. Leave the unabsorbed sample in the microfuge tube.

B5. Bind peptide from the other sample aliquots as in step 4. During the procedure, some adjustment of the volume setting on the micropipettor may be necessary.

B6. Without changing the volume setting on the micropipettor, wash the tip 4 times with 10  $\mu$ l each of solvent A, expelling the washes onto a lint-free tissue. After the last wash dab the tip well, but not to excess, on the lint-free tissue.

B7. In quick succession, transfer the ZipTip® to a 2  $\mu$ l micropipettor set to 2  $\mu$ l, aliquot 1  $\mu$ l of solvent B to a microfuge tube, and aspirate and expel the solvent into the ZipTip® 3 times to elute peptides. Aspirate the solvent into the tip once more, and expel the entire volume into a sample vial for the mass spectrometer.

The sample vial may contain LC-solvent without acetonitrile to dilute the acetonitrile in the sample for direct loading (8.5-fold dilution minimum), or the sample may be dried in a vacuum concentrator and resuspended in an appropriate volume of LC-solvent\* for loading (at least the same volume as used for elution from the tip, which can be more than 1  $\mu$ l in this case).

\*eg. 5% acetic acid, 5% MeCN

*Sep-Pak® is a registered trademark of Waters Corporation.  
ZipTip® is a registered trademark of Millipore Corporation.*