TiO_2 Mag Sepharose™

TiO_2 Mag Sepharose magnetic beads use titanium dioxide (TiO_2)-based chromatography to simplify capture and enrichment of phosphopeptides (Fig 1). TiO_2 has high affinity for phosphopeptides and provides efficient enrichment of phosphopeptides from complex samples.

TiO_2 Mag Sepharose benefits are:

- Selective enrichment of phosphopeptides
- Easy to use — visible and dense magnetic beads
- Simple protocol with elution conditions optimized for MS analysis
- Parallel handling of samples — six samples can be processed in less than one hour

Phosphorylation is a common reversible post-translational modification involved in the regulation of many essential biological processes. Phosphoproteins and phosphopeptides are usually present at very low concentrations and ionize poorly, making their detection by mass spectrometry (MS) difficult.

Phosphopeptides from, for example, trypsin-digested protein sample bind to TiO_2 while non-phosphopeptides and other components remain in the sample solution. Sample preparation procedures resulting in enrichment of phosphopeptides simplifies identification of phosphoproteins by MS. With improved sensitivity, this method allows identification of phosphoproteins down to femtomol levels.

TiO_2 Mag Sepharose is available in two pack sizes: 1 × 500 µl suitable for 10 samples and 4 × 500 µl suitable for 40 samples. Together with MagRack 6, a separation tool for handling the beads in microcentrifuge tubes, up to six samples can be processed in parallel. The rack consists of anodized aluminum housing with a detachable plastic bar containing six neodymium magnets.

TiO_2 Mag Sepharose is a complementary product to Phos SpinTrap™ Fe, a non-magnetic IMAC-based chromatography medium.

Fig 1. TiO_2 Mag Sepharose is designed for efficient small-scale enrichment of phosphopeptides.

Simple handling

TiO_2 Mag Sepharose improves ease-of-use in sample preparation of phosphopeptides. The magnetic bead format has excellent properties for small-scale experiments. The high density of the beads allows rapid capture by magnetic devices while the visibility of the beads ensures reliable collection of all target peptides bound (Fig 2). The characteristics of TiO_2 Mag Sepharose are summarized in Table 1. The products are provided with protocols optimized for downstream analysis, such as MALDI-ToF MS and LC-MS.

MagRack 6 enables preparation of up to six samples captured in 1.5 ml microcentrifuge tubes. When the tubes are placed in the rack, the magnetic beads are attracted to the magnet within a few seconds. This allows easy removal of the supernatant whereas the magnetic beads are left in the tube.

Fig 2. The high density of the beads allows rapid capture by MagRack 6 magnetic device.

Table 1. Characteristics of TiO_2 Mag Sepharose

Matrix	Paramagnetic, spherical, highly cross-linked agarose particles
Ligand	TiO_2
Particle size	37–100 µm
Working temperature	Room temperature
Storage solution	20% ethanol
Storage temperature	4°C to 30°C

Reproducible peptide enrichment: a benchmark analysis

To demonstrate efficiency and sensitivity, TiO_2 Mag Sepharose was compared with two other products. The comparison was performed in GE Healthcare's laboratories.

Parallel experiments were set up with Phos-trap™ Phosphopeptide Enrichment Kit (PerkinElmer Inc), and Titansphere™ Phos-TiO Kit (GL Sciences Inc). Titansphere Phos-TiO Kit is tip-based while TiO_2 Mag Sepharose and Phos-trap Phosphopeptide Enrichment Kit add the advantages with magnetic beads.

Two phosphorylated proteins (α-casein and β-casein) and one non-phosphorylated protein (bovine serum albumin) were reduced and alkylated with Tris(2-carboxyethyl) phosphine (TCEP) and iodoacetamide, respectively, followed by trypsin-digestion. A total of 50 pmol of each protein digest was mixed, applied to each separation medium, and used according to the procedures recommended by the manufacturer as summarized in Table 2. After enrichment, eluates were lyophilized and dissolved in 20% acetonitrile with 0.1% trifluoroacetic acid (TFA, 20 µl) and analyzed by MALDI-ToF MS. All three products enriched phosphopeptides. MS spectra with annotated phosphopeptides are shown in Figure 3A.

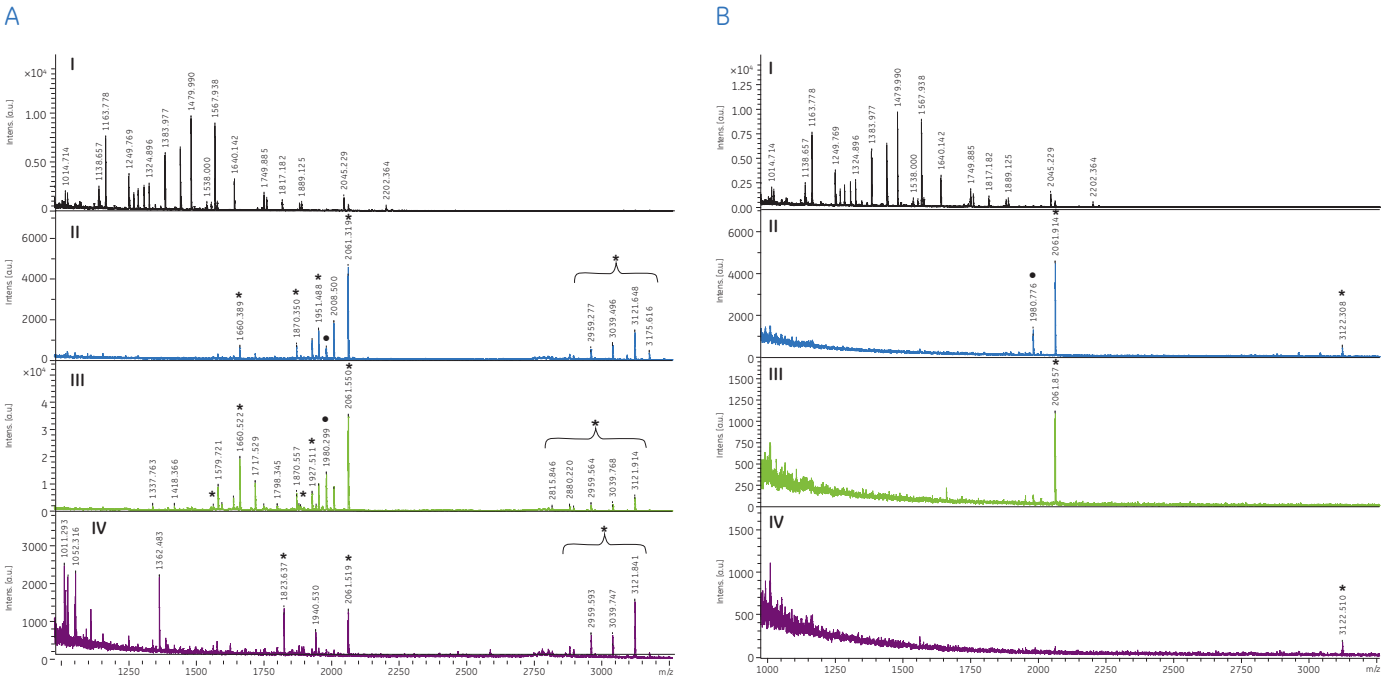
Titansphere Phos-TiO Kit detected six peptides, whereas TiO_2 Mag Sepharose found five peptides, and Phos-trap Phosphopeptide Enrichment Kit detected three peptides. Furthermore, different ratios between phosphopeptides and non-phosphorylated peptides were obtained.

The ratio obtained for TiO_2 Mag Sepharose was 2.5. The equivalent ratios for Phos-trap Phosphopeptide Enrichment Kit and Titansphere Phos-TiO Kit were 0.5 and 0.6, respectively. These results demonstrate the high selectivity for phosphopeptides using TiO_2 Mag Sepharose.

When the three eluates were diluted 100-fold, the MS spectra showed different patterns (Fig 3B). Two phosphopeptides could still be detected in the eluate from TiO_2 Mag Sepharose, while the other two eluates displayed one phosphopeptide.

Table 2. Experimental conditions

	TiO_2 Mag Sepharose	PhosTrap Phosphopeptide Enrichment Kit	Titansphere Phos-TiO Kit
Format	Magnetic medium	Magnetic medium	Prepacked tip column
Sample	Mix of tryptic fragments from bovine serum albumin, α-casein and β-casein (50 pmol each)	Mix of tryptic fragments from bovine serum albumin, α-casein and β-casein (50 pmol each)	Mix of tryptic fragments from bovine serum albumin, α-casein and β-casein (50 pmol each)
Sample volume	100 µl	100 µl	130 µl
Conditioning/equilibration	1 × 500 µl binding buffer (1 M glycolic acid, 5% TFA, 80% acetonitrile)	3 × 200 µl binding buffer from kit	1 × 20 µl solution A from kit 1 × 20 µl solution B from kit
Binding	1 × 30 min	1 × 1 min	2 × 10 min
Washing	1 × 500 µl binding buffer 2 × 500 µl washing buffer (1% TFA, 80% acetonitrile)	4 × 200 µl binding buffer from kit 1 × 200 µl washing buffer from kit	1 × 20 µl solution B from kit 1 × 20 µl solution A from kit (cycle repeated twice)
Elution	2 × 50 µl elution buffer (5% ammonia)	1 × 10 µl elution buffer from kit	1 × 50 µl of 0.5% ammonia (recycle once)



***** = phosphopeptide • = metastable phosphopeptide

Fig 3. MALDI-ToF MS analysis of trypsin-digested protein mix (50 pmol each of BSA, α-casein, and β-casein) enriched using three different chromatographic media. **(A)** Spotting from lyophilized eluates dissolved in 20 µl and **(B)** eluates diluted 100-fold before spotting. The spectra show start material **(Panel I)** and eluates from TiO_2 Mag Sepharose **(Panel II)**, Titansphere Phos-TiO Kit from GL Sciences **(Panel III)**, and Phos-trap Phosphopeptide Enrichment Kit from Perkin Elmer **(Panel IV)** respectively.

Enrichment of phosphorylated peptides from human cancer cells

Cell signaling is of prime importance for the study of various disease states in human cells, for example, different cancer cells. Signaling is often regulated by reversible phosphorylation of particular proteins on specific serine (Ser), threonine (Thr), or tyrosine (Tyr) residues and knowledge about phosphorylation of these proteins may help to understand disease progression. Tyr phosphorylation, which is low abundant, can be targeted by using antibodies in immunoprecipitation experiments.

Analysis of the phosphorylation pattern of digested proteins is improved by enrichment of phosphopeptides/fragments with TiO_2-affinity capture followed by LC-MS/MS analysis. Phosphopeptides are captured on TiO_2 independent of phosphorylation site.

In this application*, TiO_2 Mag Sepharose was used to enrich phosphopeptides from trypsin-digested proteins derived from a leukemia cancer cell line expressing an oncogene, BCR-ABL, prior to MS analysis. The aim was to map the general phosphorylation pattern.

Lysate from human cancer cells (4×10^8) was handled according to the workflow described in Figure 4. TiO_2 Mag Sepharose beads (10 µl) were equilibrated in 500 µl binding buffer. Digest corresponding to 500 µg protein in a total volume of 140 µl was incubated with the beads under mixing for 30 min. The beads were recovered, eluted, and subject to LC-MS/MS analysis.

The enriched material was compared to the protein digest before enrichment (100 µg). The number of significant peptides detected was, as expected, higher in the starting material. Phosphospeptides were, however, only detected after enrichment.

*** Acknowledgements:**
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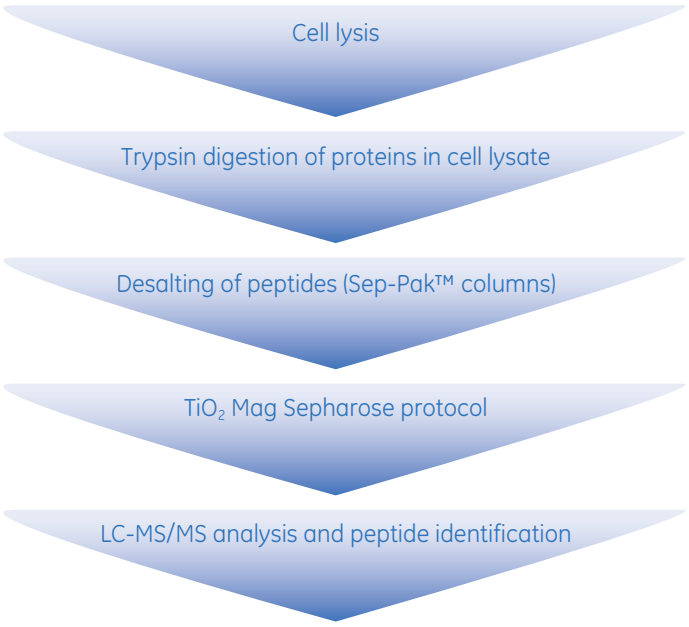

Fig 4. Workflow for preparation of phosphopeptides from leukemia cell line using TiO_2 Mag Sepharose for enrichment prior to LC/MS/MS analysis.

Table 3 lists the phosphopeptides detected by MS in the enriched peptide mixture. Of all detected peptides, 16% were phosphorylated. In total, 15 phosphopeptides and 14 phosphorylation sites were found.

To visualize the detection of one specific phosphorylation site, MS-MS spectra (both collision-associated dissociation, CAD, and electron capture dissociation, ECD) were used. The fragmentation of the IGEGTyGVVYK peptide parent ion from the cell division protein kinase 3 protein is shown in Figure 5.

This initial experiment shows promising results and demonstrates the usefulness of TiO_2 Mag Sepharose as a tool to study phosphorylation patterns. Both Ser and Tyr phosphorylations were detected.

Table 3. Enriched phosphopeptides from cancer cells

Accession number[1]	Gene name	Protein name	Phosphopeptide sequence[2]	Site	SWISS-PROT[3]
IPI00012442	G3BP1	Ras GTPase-activating protein-binding protein 1	**sss**PAPADIAQTVQEDLR	S230/S231/S232	Yes, for all 3
IPI00009032	SSB	Lupus La protein	FA**s**DDEHDEHDENGATGPVKR	S366	Yes
IPI00009032	SSB	Lupus La protein	TKFA**s**DDEHDEHDENGATGPVKR	S366	Yes
IPI00025512	HSPB1	Heat-shock protein β-1	QL**s**SGVSEIR	S82	Yes
IPI00185526	SAMSN1	SAM-domain protein SAMSN-1	SS**s**FGNFDR	S11	No
IPI00017297	MATR3	Matrin-3	RD**s**FDDRGPSLNPVLDYDHGSR	S188	Yes
IPI00184330	MCM2	DNA replication licensing factor MCM2	GLL**y**DSDEEDEERPAR	Y137(or S139)	No, S139 known
IPI00017659	RCSD1	Protein kinase substrate CapZIP	SQ**s**DCGELGDFR	S179	Yes
IPI00023503	CDK3	Cell division protein kinase 3	IGEGT**y**GVVYK	Y15	Yes
IPI00013721	PRPF4B	Serine/threonine-protein kinase PRP4 homolog	LCDFGSASHVADNDITP**y**LVSR	Y849	Yes
IPI00337465	KLC1	Isoform P of Kinesin light chain 1	A**ss**LNVLNVGGK	S546/S547	No[4]
IPI00163505	RMB39	Isoform 1 of RNA-binding protein 39	DK**s**PVREPIDNLTPEER	S136	Yes
IPI00014177	SEP2	Septin-2	IYHLPDAE**s**DEDEDFKEQTR	S218	Yes
IPI00299254	EIF5B	Eukaryotic translation initiation factor 5B	NKPGPNIE**s**GNEDDDASFK	S214	Yes
IPI00178667	TOP2A	M_r 183 000 protein/DNA topoisomerase 2	**y**LEESDEDDLF	Y1601	No

[1] International Protein Index
[2] Sequences of identified phosphopeptides; **s** indicates serine and **y** indicates tyrosine phosphorylation
[3] Phosphorylation is reported in SWISS-PROT protein database
[4] Sequence is specific for isoform P

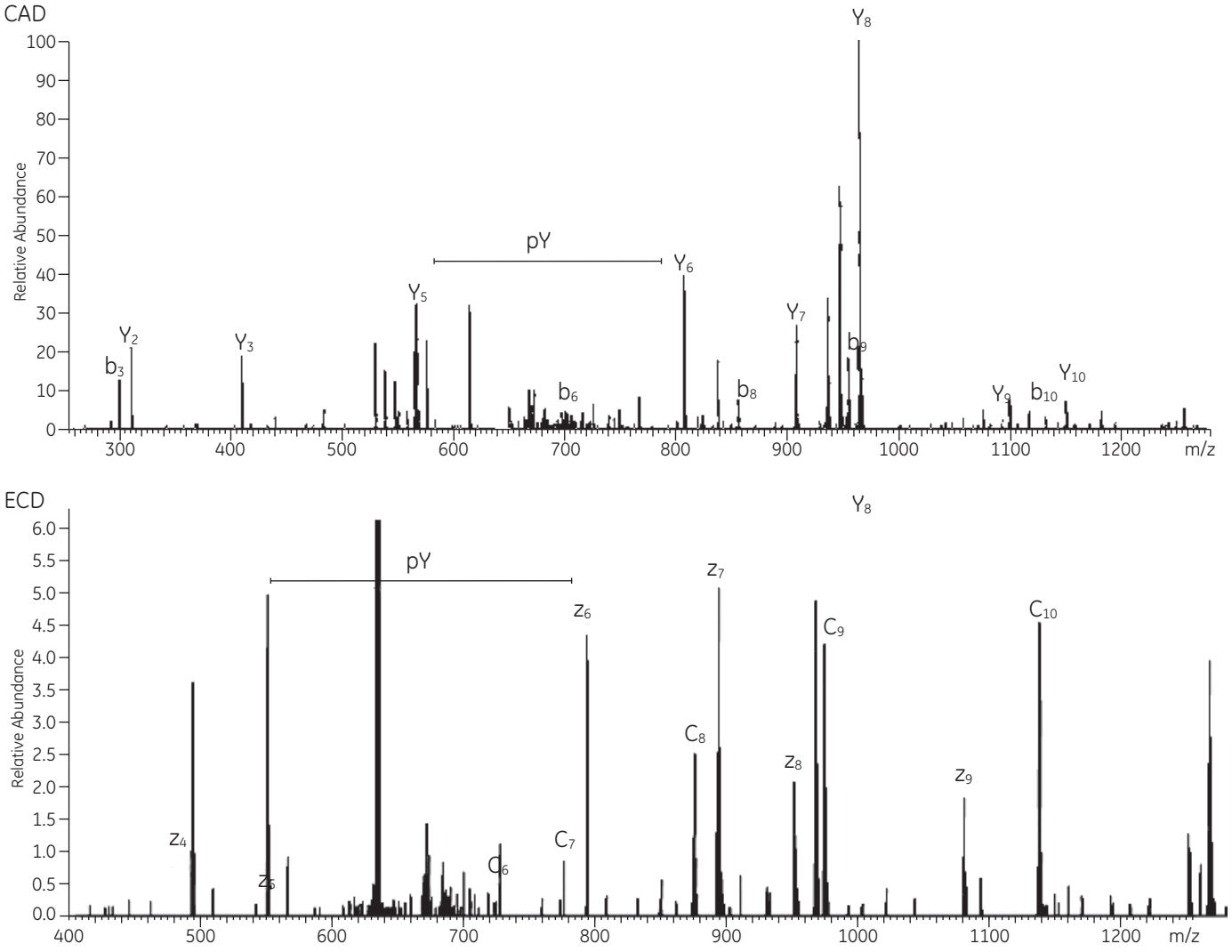

Fig 5. The parent ion of IGEGTyGVVYK was fragmented into b and y ions using CAD, and into c and z ions using ECD. The bars (pY) indicate examples of ions that matched the phosphorylation site of Y15.

Ordering information

Product	Quantity	Code no.
TiO_2 Mag Sepharose	1 × 500 µl	28-9440-10
TiO_2 Mag Sepharose	4 × 500 µl	28-9513-77

Related products	Quantity	Code no.
Phos SpinTrap Fe	2.5 ml	29-9298-81
Protein A Mag Sepharose	1 × 500 µl	28-9440-06
Protein A Mag Sepharose	4 × 500 µl	28-9513-78
Protein G Mag Sepharose	1 × 500 µl	8-9440-08
Protein G Mag Sepharose	4 × 500 µl	28-9513-79
NHS Mag Sepharose	1 × 500 µl	28-9440-09
NHS Mag Sepharose	4 × 500 µl	28-9513-80
MagRack 6	1	28-9489-64

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