Simple immunoprecipitation using paramagnetic beads

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Simple immunoprecipitation using paramagnetic beads

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Background

A major challenge in analytical scale protein purification is to selectively capture and isolate low abundant proteins from a complex sample with several thousands of other proteins. Immunoaffinity purification, or immunoprecipitation, is an ideal technique for capture and concentration of a broad range of proteins using specific antibodies.

We have tested a new type of magnetic beads (magnetite emulsified agarose) in three different applications. These beads combine the large binding capacity of Sepharose™ with magnetic properties to make it possible to work in low microlitre volumes with negligible loss of the beads.

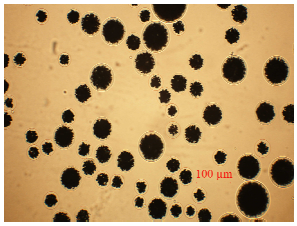

Fig 1. Microscopy picture of Mag Sepharose beads. The magnetite is centered in the middle of the particles with a thin shell of pure agarose on the surface.

Three new products have been developed, and the total protein binding capacity has been adjusted to reach a balance between capacity and the amount of antibodies needed for a typical immunoprecipitation experiment.

Characteristics

The matrix consists of paramagnetic, spherical, highly cross-linked agarose particles. The particle size is 37–100 µm.

Protein A Mag Sepharose	
Ligand	Native protein A
Binding capacity	8–17 mg human IgG/ml gel
Protein G Mag Sepharose	
Ligand	Protein G
Binding capacity	13–22 mg human IgG/ml gel
NHS Mag Sepharose	
Activated group	N-hydroxysuccinimide
Substitution degree	8–14 µmol/ml gel

Reproducible protein enrichment

The robustness was demonstrated by running 10 replicates in parallel. Human transferrin (hTf) was enriched on Protein A Mag Sepharose from a background of E. coli proteins using a polyclonal rabbit anti-human transferrin antibody. hTf was labeled with CyDye™ DIGE Fluor Cy™5 minimal dye and the eluates were analyzed by SDS-PAGE, post-stained with Deep Purple™ total protein stain. The gel was scanned using Ettan™ DIGE Imager directly after the electrophoresis. The recovery average was 53% as calculated from a standard curve of diluted start material. The average purity was 52% as determined from the Deep Purple fluorescence signal.

Medium:	25 µl Protein A Mag Sepharose 20% gel slurry
Sample:	7.5 µg/ml hTf (human Transferrin) in 5 mg/ml *E. coli* protein
Sample volume:	200 µl
Capturing antibody:	Polyclonal rabbit α-hTf
Binding buffer:	Tris buffered saline (TBS) 50 mM Tris, 150 mM NaCl, pH 7.5
Wash buffer:	TBS, 2 M urea, pH 7.5
Elution buffer:	100 mM glycine, 2 M urea, pH 2.9

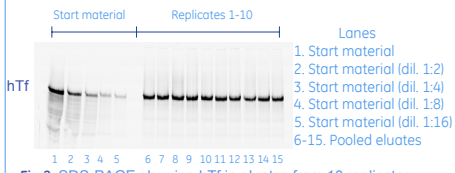

Fig 2. SDS-PAGE showing hTf in eluates from 10 replicates.

Finding low-abundant Tyr-phosphorylated proteins in complex mixtures

Proteins active in signaling pathways are normally not detectable with SDS-PAGE or MS-analysis without sample preparation due to their low abundance.
CHO-cells (7 x 10^7) grown in rich medium were used as a source of tyrosine-phosphorylated (pTyr) proteins. Pervanadate, a phosphatase inhibitor, was added two hours prior to harvest in order to prevent dephosphorylation. In a control experiment, this step was omitted.

The cells were lysed in mammalian protein extraction buffer with EDTA, Na-deoxycholat, protease inhibitors and phosphatase inhibitors. The lysate was clarified by centrifugation and diluted two-fold with binding buffer (TBS) before addition to Protein G Mag Sepharose. The pTyr proteins were eluted with 100 mM phenylphosphate (2 × 5 minutes in 37˚C). The eluates were digested with trypsin and analyzed by LC-MS/MS.
In the eluate, 76 potential tyrosine phosphorylated proteins were identified. Of these hits, 54 were exclusively found in the pervanadate-treated cells and were neither found in the control cells nor in the start material. Some of these proteins such as Caveolin-1, FAK, and SHC are known to be involved in focal adhesion pathways. These proteins may be involved in the regulation of the actin cytoskeleton. Other proteins that are involved in cell motility or cell survival were also found. In the control samples (untreated cells), only 22 proteins were detected, mainly high abundant enzymes and ribosomal proteins. This example clearly demonstrates a simple and efficient capture of pTyr proteins.

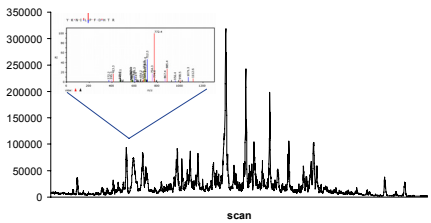

Fig 3. MS analysis of eluted proteins after immunoprecipitation of pTyr proteins from pervanadate-stimulated cells. Before analysis the proteins were cleaved with trypsin. An example MS/MS spectrum of a pTyr peptide is shown and was identified as PTPn11.

Enrichment of plasminogen from human plasma

NHS Mag Sepharose is the obvious first choice when searching for other proteins than IgG in plasma. Endogenous immunoglobulins may bind to remaining free ligands of Protein A Mag Sepharose or Protein G Mag Sepharose.
Human plasma contains a vast number of proteins and can be difficult to work with due to the great range of protein concentrations. Plasminogen was enriched from human plasma using an anti-plasminogen mouse IgG1 monoclonal antibody covalently coupled to NHS Mag Sepharose. The fractions were analyzed on SDS-PAGE and plasminogen was further identified by LC-MS/MS. This medium abundant protein was enriched more than thousandfold. Analysis of this gel band by MALDI PMF after in-gel digestion with trypsin confirmed the identity of human plasminogen.

Medium:	25 µl NHS Mag Sepharose 20% gel slurry
Sample:	Human plasma
Sample volume:	150 µl
Antibody:	Monoclonal mouse anti-plasminogen, subtype IgG1
Coupling buffer:	150 mM triethanolamine, 500 mM NaCl, pH 8.3
Binding buffer:	TBS, 50 mM Tris, 150 mM NaCl, pH 7.5
Wash buffer:	TBS, 2 M urea, pH 7.5
Elution buffer:	0.1 M glycine/HCl, 2 M urea, pH 2.9

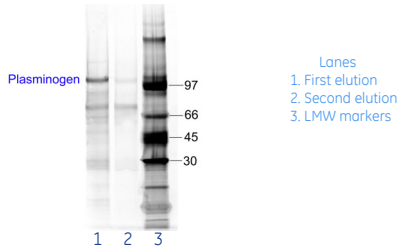

Fig 4. SDS-PAGE showing a protein close to the 97kDa LMW marker.

Conclusions

Mag Sepharose technology provides the following benefits:

- **Magnetic beads allow simple and reproducible handling**
- **Successful capture of low abundant proteins**
- **Efficient concentration and enrichment of cell signaling proteins from mammalian cells**

imagination at work