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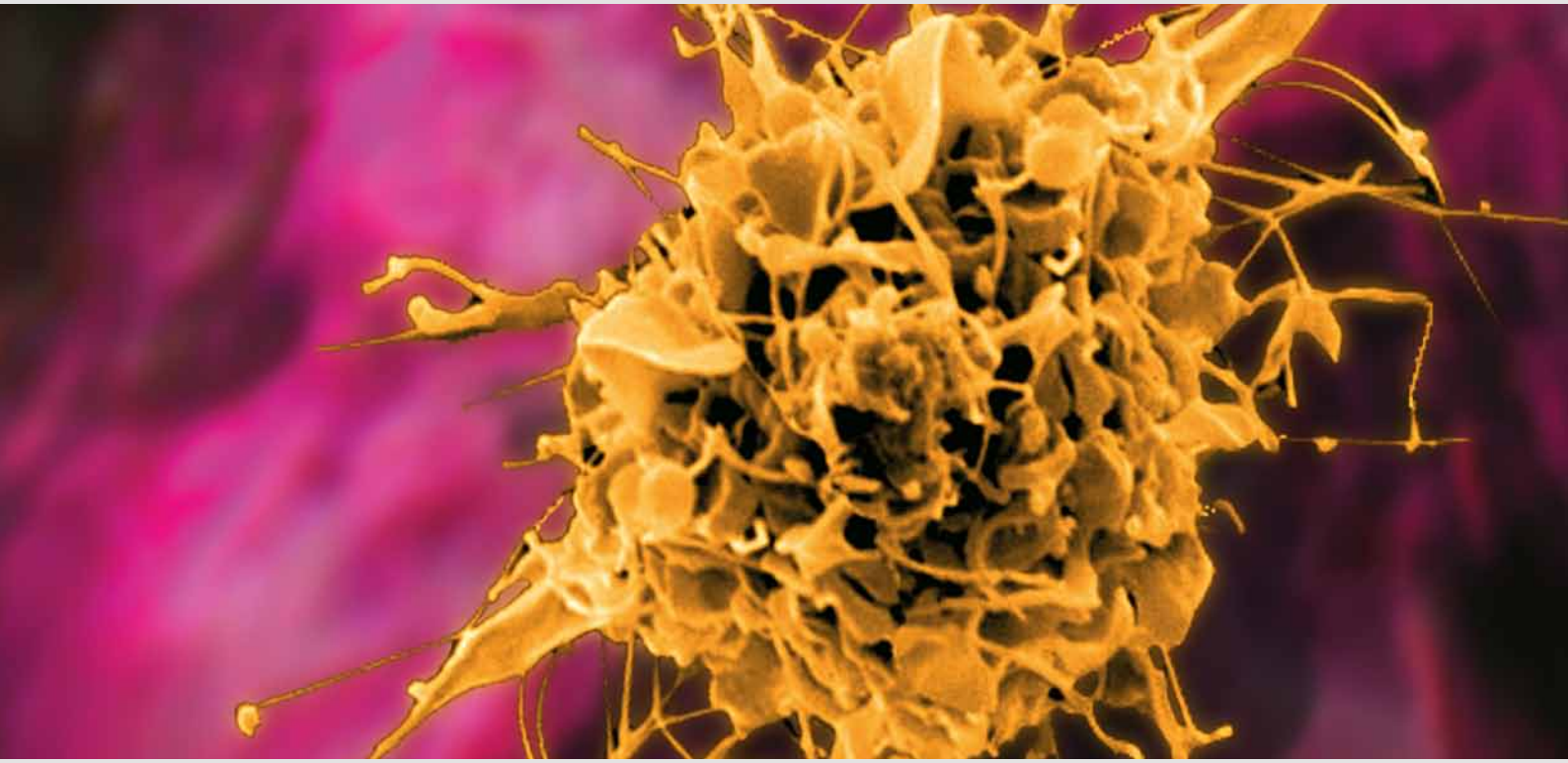

As reported by David Mulholland and co-workers, MACS® Column Technology is the method of choice for co-immunoprecipitation, chromatin immunoprecipitation, and isolation of DNA-binding proteins. It allows for faster as well as more efficient isolations when compared to standard immunoprecipitation techniques.

Enhanced target identification of the androgen receptor in mammalian cells and tissue



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Introduction

Androgens and dihydrotestosterone (DHT) control the development, differentiation, and function of male reproductive tissue. Androgen also affects other organs and tissues, such as skin, brain and bone marrow. The principle action of androgen is to regulate gene expression via the androgen receptor (AR), which belongs to the superfamily of nuclear receptors. Nuclear receptors are ligand-inducible transcription factors that mediate the signals of a broad variety of fat-soluble hormones.

In this report, super-paramagnetic µMACS Protein G MicroBeads together with MACS Column Technology were used to immunoprecipitate activated AR as well as the associated ligand Beta-Catenin. Further, the Protein G MicroBeads were used for chromatin immunoprecipitation (ChIP) to identify the binding site of AR. Using µMACS Streptavidin MicroBeads we isolated a specific DNA-bound protein, which was identified as a phosphoprotein.

MACS products

µMACS Protein G MicroBeads	# 130-071-101
µMACS Streptavidin MicroBead Kit	# 130-074-101
µ Columns	# 130-042-701
µMACS Starting Kit	# 130-042-602

Materials and methods

Immunoprecipitation from LNCaP cultured cells. LNCaP cells were subjected to lysis using either RIPA buffer (0.1 % SDS, 150 mM NaCl, 50 mM Tris pH 8.0) or NP40 lysis buffer (1 % NP-40, 150 mM NaCl, 50 mM Tris pH 8.0). A total of 500 µg of cell lysate per IP were used to immunoprecipitate AR using anti-AR antibodies (Pharmingen) with µMACS Protein G Microbeads (Miltenyi Biotec) or Protein A/G plus agarose beads (Santa Cruz). Proteins were detected by Western blotting using anti-AR and anti-Beta-Catenin (Santa Cruz) antibodies.

Chromatin immunoprecipitation from LNCaP cultured cells and tumors. LNCaP prostate cancer cells, grown in vitro, were treated with 10 nM R1881 (DHT) for 48 hours, were crosslinked with 1 % formaldehyde, and placed on a shaker for 10 min at room temperature. Excised tumors were minced in cold phosphate buffer followed by immersion fixation with formaldehyde (60 min) for 15 min at 37 °C. In both instances, Chromatin/protein crosslinking was stopped by adding 0.125 M glycine and rocking for 5 min. Cultured LNCaP cells were scraped from the dish or minced tumor cells were collected and centrifuged in cold PBS (protease inhibitors optional) and resuspended in cold cell lysis buffer with inhibitors (5 mM PIPES pH 8.0, 85 mM KCl, 0.5 % NP40, Roche complete protease inhibitors) for 10 min. Nuclei were pelleted by centrifuging at 5,000 rpm for 5 min. The cytosolic portion was removed and resuspended in 300 µL nuclear lysis buffer with inhibitors (50 mM Tris-Cl pH 8.1, 10 mM EDTA, 1 % SDS, protease inhibitors) for 10 minutes. Chromatin was sonicated to an average of 500 bp (assessed by gel) by using 5 x 10 seconds pulses at ~ 30 % max. setting on a sonicator in a 15 mL conical tube. In order to reduce SDS concentration to ~0.1 %, lysate was diluted ten-fold (i.e. 150 µL of lysate to 1500 µL) with IP dilution buffer (0.01 % SDS, 1 % NP40, 1.2 mM EDTA, 16.7 mM Tris-Cl pH 8.1, 167 mM NaCl). To clear non-specific binding proteins and DNA, supernatants were pre-cleared for 30 min using 20 µL of the respective protein G beads and sheared salmon sperm DNA. Supernatants were then microfuged for 5 min at 15,000 rpm and lysate was harvested. Either 2.5 µg of anti-AR antibody or an equivalent amount of non-immune control IgG (Santa Cruz) were added to the lysate and inverted overnight. The lysate was combined with 20-30 µL of µMACS Protein G MicroBeads and incubated for 1 hour.

The complete volume of cell lysate/µMACS MicroBeads was pipetted onto a MACS Column, allowed to flow through, and washed 4x500 µL with ChIP wash buffer (100 mM Tris-Cl pH 8.0, 500 mM LiCl, 1 % NP40, 1 % deoxycholic acid). ChIP product was then eluted by applying 2x150 µL of ChIP elution buffer (50 mM $NaHCO_3$, 1 % SDS) to each column. To reverse the crosslinking reaction, 1 µL of RNase A (10 µg/µL) and 5 M NaCl (0.3 M final) was added to the eluate and incubated in a 67 °C waterbath for 4 hours. DNA was precipitated by adding 2.5 volumes of ethanol and incubation at -20 °C overnight. DNA was concentrated by microfuging at 14,000 rpm at 4 °C for 15-20 min, washed with ethanol, and resuspended in 175 µL of TE. Residual salts were removed by a phenol/chloroform clean-up step. 750 µL of EtOH and 5 µg of glycogen (painted) were added and suspensions were again precipitated overnight at -20 °C. Samples were microfuged at 14,000 rpm for 20 min (4 °C) and DNA was resuspended in 30 µL of TE.

PCR was performed on samples using 1-2 µL of sample for 35 cycles at 58 °C. For PCR, oligonucleotide primers specific for the prostate-specific antigen (PSA) gene were used:
5'-TCTGCCTTTGTCCCCTAGAT-3',
5'-AACCTTCATTCCCCAGGACT-3'
and others within the gene for further verification of pull-down specificity.

Results

In order to evaluate the efficiency of µMACS MicroBeads, we used prostate cancer cells (LNCaP) cultured *in vitro*. LNCaP cells were treated with dihydrotestosterone (DHT), the physiological ligand for the androgen receptor (AR), to promote activation of AR transcription and to complete nuclear translocation of the receptor within 60 minutes. Utilizing the µMACS Protein G MicroBeads, we set out with two initial goals in mind: (1) to compare the ability of the MicroBeads with conventional Protein A/G agarose beads in immunoprecipitation of the AR and (2) to co-immunoprecipitate a known AR binding partner such as Beta-Catenin[1].

Immunoprecipitation results (fig. 1) showed that using µMACS Protein G MicroBeads at half the bead volume of the agarose beads, we were able to immunoprecipitate 3-4 times more AR. Under NP-40 lysis conditions, we validated that the µMACS MicroBeads were also more efficient in isolating Beta-Catenin (fig. 2).

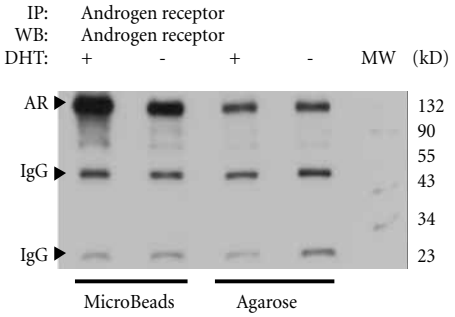

Figure 1: Western Blot of immunoprecipitated AR using anti-AR antibody. AR was immunoprecipitated from DHT-stimulated (lane 1, 3) or unstimulated LNCaP cells (lane 2, 4) with µMACS Protein G MicroBeads (lanes 1-2) or with Protein A/G agarose beads (lanes 3-4).

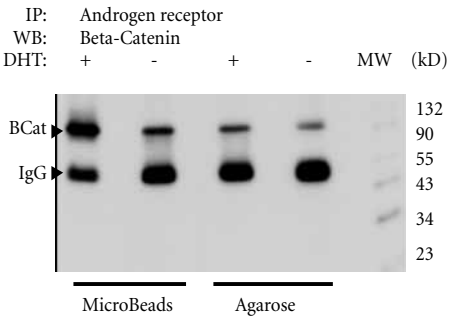

Figure 2: Western Blot of AR-co-immunoprecipitated Beta-Catenin using anti-Beta-Catenin antibody. AR was immunoprecipitated from DHT-stimulated (lane 1, 3) or unstimulated LNCaP cells (lane 2, 4) with µMACS Protein G MicroBeads (lanes 1-2) or with Protein A/G agarose beads (lanes 3-4)

The AR/Beta-Catenin interaction is known to be ligand-sensitive as is evident from the result obtained using the agarose beads. Immunoprecipitation with µMACS Protein G MicroBeads showed a four- to five-fold increase in ligand dependency, as opposed to a two-fold increase as seen with the agarose beads. The ability for Protein G MicroBeads to isolate complexes prompted us to evaluate the efficiency of the MicroBeads with more difficult isolation techniques.

Chromatin immunoprecipitation (ChIP), a technique that isolates chemically fixed protein/DNA complexes by standard immunoprecipitation procedures, is a powerful tool for studying protein-DNA interactions. A fundamental question in prostate cancer research revolves around the discovery of novel transcriptional regulators that may be directly bound to promoter regions of the PSA gene. Assaying for transcriptional regulator/DNA complexes in solid prostate tumors is likely to be an area of abundant discovery. After isolation of the complexes, the cross-linking is reversed, the DNA is ethanol precipitated and subsequently identified by PCR. In the presence of androgen, the AR translocates to the nucleus and binds co-operatively to promoter regions. LNCaP cells not exposed to androgen should theoretically not have large amounts of the target PSA gene bound to the AR. One of the technical challenges of performing ChIP experiments is to produce a clear positive PCR signal while minimising the presence of non-specific PCR products. In work done previously, we used a panel of AR antibodies in combination with agarose beads and were consistently able to generate a moderate level of PCR product using primers targeted towards the AR binding regions of the PSA gene in androgen-induced LNCaP cells.

However, PCR products were also amplified from the negative control with yields approaching 60 % of those of the androgen-induced samples. When we used µMACS MicroBeads, we not only observed enhanced immuno-precipitation under fixed conditions but we also observed reduced levels of non-specific PCR product (fig. 3).

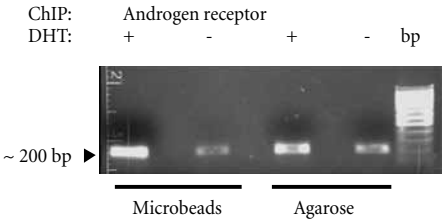

Figure 3: PCR reaction of the PSA gene using ChIP obtained DNA from cultured cells. Immunoprecipitation was carried out with µMACS Protein G MicroBeads (lanes 1-2) or with Protein A/G agarose beads (lanes 3-4). Anti-AR antibody (lane 1, 3) or as a control anti-IgG (lane 2, 4) were used for ChIP.

A more difficult extension of ChIP is to precipitate target genes from tissues. Using LNCaP cells, that were grown in culture, injected subcutaneously to the dorsal side of mice and harvested as a capsulated tumor, we evaluated the use of the µMACS MicroBeads. When using agarose beads, we had considerable difficulty reducing the amount of non-specific PSA gene product signal in the IgG negative control (fig. 4).

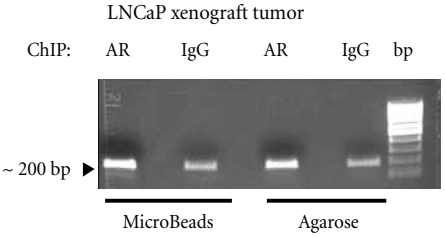

Figure 4: PCR reaction of the PSA gene using ChIP obtained DNA from tumor cells. Immunoprecipitation was carried out with µMACS Protein G MicroBeads (lanes 1-2) or with Protein A/G agarose beads (lanes 3-4). Anti-AR antibody (lane 1, 3) or as a control anti-IgG (lane 2, 4) were used for ChIP.

In many cases our experimental signal was the same intensity as the negative control. Using Protein G MicroBeads we were able to reduce the non-specific product significantly with an equal or lower volume of beads.

Given the ability of µMACS Protein G MicroBeads to specifically precipitate protein species in greater amounts than agarose beads, we decided to use MACS Technology for protein discovery. We designed a biotinylated DNA probe to attempt to isolate LNCaP nuclear protein associated specifically with a promoter region in the PSA gene.

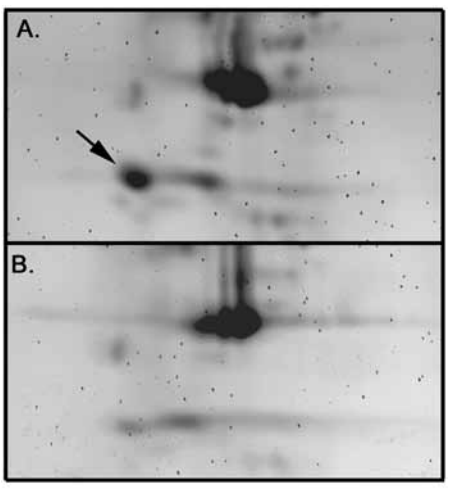

Figure 5: 2D-gel electrophoresis of proteins bound to the biotinylated target DNA (A) or a non-specific biotinylated control DNA (B).

To isolate DNA binding proteins, µMACS Streptavidin MicroBeads were used to pull biotinylated DNA/protein complexes from LNCaP lysate. Following elution, the samples were separated using 2D gel electrophoresis and stained with Cypro red (Molecular Probes) (fig 5). Our DNA probe clearly shows a major protein product (1, arrowhead) while a non-specific DNA probe (2) shows little sign of a corresponding species. The labeled spot was excised from the gel and sequenced by mass spectrometry (MALDI-TOF) and subsequently identified as a phosphoprotein.

Discussion

Several unique features of the µMACS MicroBeads, together with the Column Technology, likely contribute to their ability to increase the yield and specificity of a precipitated protein. Among these are the high surface area-to-volume ratio, the colloidal nature of the MicroBeads as well as their super-paramagnetic property. When we used conventional Protein A/G agarose beads we were, at best, only able to achieve semi-quantitative analysis. At only a slight cost increase compared to the agarose beads, the combination of the Microbeads with the µ Columns allowed us to achieve increased efficiency and, therefore, evaluate our immunoprecipitations in a more quantitative manner. Increased yields may also ease protein identification and even facilitate protein discovery.

References:

1. Mulholland, D.J. *et al.* (2002) J. Biol. Chem. 277: 17933-17943.