MACS molecular

Isolation of RNA binding proteins

µMACS Streptavidin Kit Order No. 130-074-101

Index

1. Background

1.1 Reagent and instrument requirement

2. Protocol

2.1 Biotinylated oligonucleotide

2.2 Generation of mRNA transcripts

2.3 Labeling of the in vitro transcribed mRNA with a 3´ biotinylated oligonucleotide and the µMACS Streptavidin MicroBeads

2.4 Preparation of a crude cellular extract / protein extract

2.5 Incubation of the magnetically labeled mRNA with the protein extract

2.6 Preparation of the column

2.7 Separation

2.8 Elution

2.9 Analysis

Isolation of RNA binding proteins

1. Background

The regulation of gene transcription and translation into proteins is an expanding research field, for example with the aim to identify critical points in the development of cancer and other diseases. The regulation of gene expression also plays a major role in the processes of differentiation during development.

Gene expression is controlled at two levels: at the level of transcription and the level of translation. The transcription itself is accomplished by the binding of proteins (transcription factors) to specific regulatory DNA sequences, the primary mRNA is then modified during posttranscriptional processing. Equally important to over-all gene expression is the control of translation, including the controlling of the mRNA level in the cell. During the synthesis of proteins at the ribosomes, as well as during the posttranslational modification of the protein itself, cell type specific factors are involved in the regulation of protein expression.

Isolation of RNA binding proteins represents an important step in understanding RNA degradation, where the availibility of mRNA is strictly regulated. One such mechanism regulating mRNA levels in the cell involves controlling the half-life of various mRNA molecules, thereby ultimately controlling the transcript availability for translation processes.

This protocol was established for the isolation of Mating Factor A2 mRNA binding protein from yeast, but can be easily adapted for the isolation of other mRNA binding proteins.

▲ Critical parameters for successful isolations of mRNA binding proteins are mainly pH, the salt concentration of the Binding/Washing Buffer and the temperature for binding the biotinylated probe to the target mRNA.

Generally, attempts to isolate mRNA binding proteins are only conducted after evidence has been accumulated that the mRNA sequence in question does specifically interact with proteins. This evidence is usually obtained using mobility shift assays and UV cross-linking analysis.

1.1 Reagent and instrument requirement

- µMACS Streptavidin Kit
- µMACS Separator

Additionally required material:

- Buffer for binding of biotinylated oligonucleotide to the full length mRNA:
  10 mM HEPES pH 7.0, 50 mM KCl
- Binding/Washing Buffer:
  10 mM HEPES pH 7.0
  50 mM KCl
  10 % glycerol
  1 mM EDTA
  1 mM DTT
  0.5 % Triton X-100
  0.15 µg/ml yeast tRNA
  RNase inhibitor
- Elution buffer:
  Binding/Washing Buffer supplemented with 1 M NaCl
- biotinylated oligonucleotide
- mRNA transcripts

2. Protocol

2.1 Biotinylated oligonucleotide

We recommend using an HPLC-purified, 5‘- or 3‘-biotinylated ss-oligonucleotide (here a 3´ labeled oligonucleotide is used), which is commercially available (local suppliers can be found via the internet). The oligonucleotide must be complementary to the in vitro transcribed mRNA and should be at least 25 - 30 bases long.

2.2 Generation of mRNA transcripts

mRNA transcripts, which bind the RNA binding protein, are generated by in vitro transcription with T7 RNA polymerase in the presence of RNase inhibitors. For control purposes, we suggest generating a second mRNA transcript with mutated or deleted binding sites. For in vitro transcription of mRNA, there are established protocols (e.g. see Sambrook et al. „Molecular Cloning“) as well as complete kits from different suppliers (e.g. Roche) available.

2.3 Labeling of the in vitro transcribed mRNA with a 3´ biotinylated oligonucleotide and the µMACS Streptavidin MicroBeads

1. Mix 500 pmol of the biotinylated oligonucleotide with 400 pmol of mRNA transcripts in 10 mM HEPES pH 7.0, 50 mM KCl. The total volume should not exceed 900 µl.

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2. Incubate for 1 hour at room temperature.

   ▲ **Note:** Increased temperatures up to 60 °C may be used to enhance the stringency of binding.
   If the mRNA itself tends to form secondary structures, we recommend incubating the mRNA for 2-3 minutes at 95 °C before hybridization with the biotinylated oligonucleotide.

3. Add 100 µl µMACS Streptavidin MicroBeads and incubate for 5 minutes at room temperature to magnetically label the mRNA.

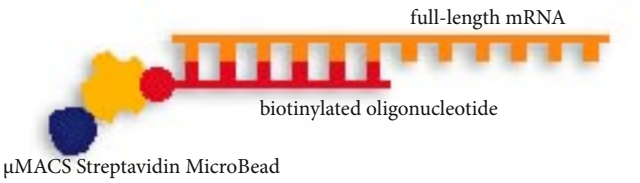


Figure 1: The magnetically labeled probe complex consisting of the in vitro transcribed mRNA labeled with a biotinylated oligonucleotide and the µMACS Streptavidin MicroBead.

2.4 Preparation of a crude cellular extract / protein extract

Prepare a crude protein extract from the cells (in the presence of protease inhibitors). To reduce possible background binding, a preclearing step by adsorption of proteins, which may bind unspecifically to the in vitro transcribed mRNA, may be helpful.

▲ **Note:** If your protein of interest does not bind to heparin, the protein extract can be cleared by using heparin-agarose chromatography. Heparin binds non-specifically to many mRNA binding proteins. For heparin-agarose chromatography, 5 ml heparin-agarose is washed three times with 5 ml of Binding/Washing Buffer. After removing the supernatant, a mixture of 1 ml crude protein extract and 4 ml Binding/Washing Buffer is added to the heparin-agarose. The mixture is rotated for 1 hour at 4 °C, and the protein extract is decanted to a fresh tube.

2.5 Incubation of the magnetically labeled mRNA with the protein extract

The protein extract is mixed with the magnetically labeled mRNA probe (see 2.3). In addition protease inhibitors may be added. Incubate for 1 h at 4°C on a rocking platform.

2.6 Preparation of the column

Place a µColumn in the magnetic field of the µMACS Separator. Prepare the column by applying 100 µl of equilibration buffer for protein applications (supplied with the kit) on top of the column followed by washing with 2 x 100 µl of Binding/Washing Buffer.

2.7 Separation

Apply the binding reaction performed in step 2.5 on top of the column matrix and let it pass through. The magnetically labeled mRNA-protein complexes are retained on the column. Rinse the column with 4 x 200 µl of Binding/Washing Buffer to remove non-specifically bound proteins.

▲ **Note:** You may increase the salt concentration of the Washing Buffer for more stringent washing steps. However, too high salt concentrations may cause the loss of the protein of interest.

2.8 Elution

Elute retained mRNA binding protein(s) from the separation column by adding 200 µl of Binding/Washing Buffer supplemented with 1 M NaCl, while the column remains in the magnetic field. Collect all eluted drops. If the eluate should be more concentrated, collect the second to fourth drop (the first drop usually does not contain any protein).

2.9 Analysis

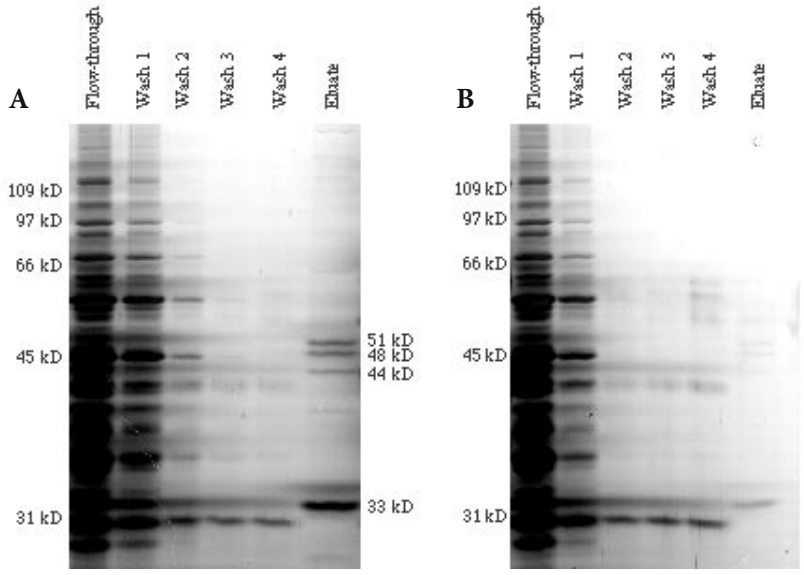
Collect washing fractions as well as the eluate and use 20 µl of each for an analysis by SDS-PAGE. We recommend silver staining the gel for maximum sensitivity.

Alternatively, Western blotting for analysis is also possible after SDS-PAGE, if specific antibodies for the separated proteins are available.

Warning

Reagents contain sodium azide. Sodium azide yields hydrazoic acid under acid conditions, which is extremely toxic. Azide compounds should be diluted with running water before discarded. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

Isolation of Mating Factor A2 mRNA binding proteins from yeast



The figure shows a silver stained SDS gel. Crude cellular extract from yeast was precleared by heparin agarose chromatography and subsequently incubated with magnetically labeled full length Mating Factor A2 mRNA (A) or as a control with magnetically labeled mutant mRNA (B), missing the binding sites for specific Mating factor A2 mRNA binding proteins (description of the magnetically labeled complex see section 2.3). Four Mating Factor A2 mRNA binding proteins with apparent molecular weights of 33, 44, 48 and 51 kD were isolated by using this approach (Courtesy of Dr. Allan Albig, Washington State University, USA).

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