MACS molecular

Isolation of specific transcripts/RNAs

µMACS Streptavidin Kit Order No. 130-074-101

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1. Background

For isolation of transcripts of eukaryotic cells, generally the whole spectrum of expressed sequences is isolated first by oligo(dT)-hybridization of the poly(A)tail. Afterwards the population of transcripts or single mRNA molecules are analyzed in detail. The direct isolation of a specific transcript is possible by hybridization of a short DNA probe. For transcripts which may lack a poly(A)tail, for example some viral transcripts, an isolation is only possible by using specific DNA probes without prior poly(A) mRNA enrichment.
This protocol describes the procedure for the isolation of a specific transcript (mRNA with or without poly(A) tail) using a biotinylated DNA probe and µMACS Streptavidin MicroBeads. Critical parameters for successful separations are mainly the hybridization (annealing) temperature for optimal binding of a transcript by a specific DNA oligonucleotide, the salt concentration of the washing buffer, and the temperature while eluting (see below for details).

1.1 Reagent and instrument requirement

- **Lysis Buffer,** i.e. from µMACS mRNA Isolation Kit (# 130-075-201, small scale or # 130-075-101, large scale)
- **TE Buffer:** 10 mM Tris/HCl pH 8.0, 1 mM EDTA
- **TEN Buffer:** 10 mM Tris/HCl pH 8.0, 1 mM EDTA, 100 mM NaCl
- **Equilibration Buffer for nucleic acid applications** (supplied with the µMACS Streptavidin Kit)
- **µMACS Separator** (# 130-042-602)
- **MACS MultiStand** (#130-042-303)
- **µ Columns** (# 130-042-701)
- **µMACS Streptavidin Kit** (# 130-074-101)
- (Optional) µMACS mRNA Isolation Kit (# 130-075-201, small scale or # 130-075-101, large scale)
- (Optional) Lysate Clear Columns (supplied with the µMACS mRNA Isolation Kit)

2. Protocol

2.1 Generation of the biotinylated capture DNA

To bind specific transcripts, a corresponding single stranded DNA oligonucleotide must be synthesized, which is complementary to the transcript of interest. The sequence must be specific for the transcript of interest. The length of the oligonucleotide should comprise at least 25-30 bases. Shorter oligonucleotides fail to isolate specific transcripts. The oligonucleotide must contain 1-2 biotin residues (5' and/or 3') and should be HPLC purified.

- Dilute the capture DNA in TE Buffer to a final concentration of 0.5 µg/µl.

2.2 Enrichment of transcripts from cells

For best results, transcripts should be isolated and purified before magnetic labeling. For mRNA with a poly(A) tail, use the **µMACS mRNA Isolation Kit** (# 130-075-201, small scale or # 130-075-101, large scale).

Alternatively, or if no poly(A) tail is present, specific transcripts may be isolated directly from a cell lysate:

1. Harvest the cells and centrifuge in a 1.5 ml tube.
2. Remove the entire supernatant and resuspend the cells completely by flicking the tube.
3. For lysis of the cells, add 1 ml of **Lysis Buffer** (i.e. from **µMACS mRNA Isolation Kit**) to a maximum of 10^7 cells and lyse the cells by vigorous vortexing for 3 minutes.

   ▲ **Note:** If the lysate is very viscous or contains visible cell debris, mechanical shearing must be performed. Therefore, the lysate should be forced one to five times through a needle (23G-20G) attached to an appropriate syringe. The foam can be reduced by centrifugation. The Lysis Buffer contains agents which inactivate RNases, thus no further RNase inhibitors are needed.
4. The lysate is now applied onto a **Lysate Clear Column** (supplied with the **µMACS mRNA Isolation Kit**). Use the flow-through for the isolation of the specific transcript.

2.3 Binding of specific transcripts to the biotinylated capture DNA

The successful isolation of a specific transcript is mainly dependent on the annealing temperature, which should allow an optimal binding of the biotinylated capture DNA to the transcript. The annealing temperature may be estimated by using the following formula which calculates the melting temperature for a DNA/RNA hybrid:

Estimation of the annealing temperature:

$$T_M = 67 + 16.6 \log_{10}([Na^+]/(1.0+0.7[Na^+])) + 0.8(\%(GC)) - 500/n$$

where $[Na^+]$ is the concentration of Na^+ ions in mol/l, *(%(GC))* is the percentage of GC in the duplex, and *n* is the length of the duplex.

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Use an annealing temperature of about 10°C below the calculated melting temperature for a catching primer with one biotin, and an annealing temperature of about 15°C below the calculated T_M for a catching primer with two biotins. If no transcript can be isolated, decrease the temperature by 2-4°C; if unspecific transcripts are co-purified with the transcript of interest, increase the annealing temperature by 2-4°C.

Example for the estimation of the annealing temperature:
$[Na^+]$ = 0.1 mol/l
%(GC) = 50
n = 25
$\Rightarrow T_M = 69.9°C \Rightarrow$ use an annealing temperature of about 60°C for a primer with 1 biotin or 55°C for a primer with 2 biotins.

1. **Transcripts purified by µMACS mRNA Isolation Kit:** Adjust the volume of the transcript solution to 200 µl by adding TEN buffer. Denature for 5 minutes at 75°C.
2. **Whole lysate:** Denature the whole lysate for 5 minutes at 75°C.
3. Adjust to the calculated annealing temperature and add 0.5 µg (1 µl) of the biotinylated capture DNA (see "Generation of the biotinylated capture DNA)). The final volume should not exceed 900 µl.
4. Vortex and incubate for 5 minutes. For rare transcripts, a longer incubation time may be necessary.

   ▲ **Note:** The amount of the capture DNA depends on the frequency of the desired transcript and should be used in excess to the transcript. Do not use more than 50-100 pmol of capture DNA (about 0.5 µg) to prevent loss of transcripts.

2.4 Preparation of the µ Column

1. Place a µ Column in the magnetic field of the µMACS Separator.
2. Prepare the column by applying 100 µl of **Equilibration Buffer for nucleic acid applications** (supplied with the µMACS Streptavidin Kit) on top of the µ Column, followed by washing with 2x200 µl of either **Lysis Buffer** (high salt) or **TEN Buffer** (low salt).

   ▲ **Note:** The amount of salt influences the binding reaction. Higher salt concentrations lead to a lower specificity between DNA/RNA duplexes.

2.5 Magnetic labeling

Magnetic labeling of the DNA-transcript complex

1. After hybridization, add 100 µl of **µMACS Streptavidin MicroBeads**. For best results, the dilution of the µMACS Streptavidin Microbeads should be no more than 1:10.
2. Vortex and incubate for 2 minutes at the annealing temperature. Immediately proceed to separation.

2.6 Magnetic separation

1. Apply the binding solution containing the magnetically labeled hybridization complexes to the µ Column and let it pass through. The magnetically labeled complexes are retained in the column.
2. Rinse the column with 5x200 µl of **TE Buffer**.

   ▲ **Note:** The composition of the Buffer for washing may be adjusted (e.g. salt concentration) to achieve optimal wash conditions; higher salt concentrations lead to a lower specificity between DNA/RNA duplexes. The temperature of wash buffers may be increased to 37°C.
3. For elution of the bound transcript, preheat RNase-free H_2O to 80°C.
4. Pipette 150 µl of the preheated H_2O on top of the µ Column. The flow-through will contain the isolated transcript. To collect a more concentrated solution, we recommend to **collect drops 2 to 4** (the first drop usually does not contain any RNA).

2.7 Analysis

If a part of the transcript sequence is known, the isolated transcript may be analyzed by a RT-PCR reaction and agarose gel electrophoresis, followed by sequencing. In the case of an unknown sequence, the poly(A)-RNA can be converted into cDNA using oligo(dT) primer and Reverse Transcriptase. The length of transcripts may be analyzed by 3'-RACE PCR.
Viral transcripts may lack a poly(A) tail. These transcripts can be polyadenylated ("poly(A) tailing") using Poly(A) polymerase.