MACS molecular

Subtractive Hybridization

µMACS Streptavidin Kit Order No. 130-074-101

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1. Background

Developmental and pathological processes often reflect qualitative and quantitative changes in gene expression. The identification of up- or downregulated genes is crucial for the understanding of those processes. The most powerful tools in identifying such genes are differential display, microarray hybridization and subtractive hybridization.
Using subtractive hybridization, it is possible to isolate and identify mRNAs that differ in abundance between two pools. The molecules of one mRNA pool are labeled, e.g. by cDNA synthesis with a biotinylated primer. The mRNA of the second pool (target mRNA) is then hybridized to the cDNA of the first pool (subtractor cDNA), and both labeled cDNA and cDNA/mRNA hybrids are immobilized. The non-labeled mRNA molecules representing differentially expressed genes are isolated.

1.1 Reagent and instrument requirements

- **Hybridization buffers:**
  20 x SSC: 3 M NaCl, 0.3 M Na-citrate, pH 7.0
  20 x SSPE: 3 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4
- (Optional) **µMACS mRNA Isolation Kits** (# 130-075-101, large scale or # 130-075-201, small scale)
- **µMACS Separator** (# 130-042-602)
- **MACS MultiStand** (# 130-042-303)
- **µ Columns** (# 130-042-701)
- **µMACS Streptavidin Kit** (# 130-074-101)

2. Protocol

This protocol is a general outline of how to perform a subtractive hybridization. The reaction conditions (e.g. hybridization temperature, hybridization time and salt concentration of the buffers) will need to be adapted to your individual application.

- Isolate the two mRNA pools of interest (e.g. with the **µMACS mRNA Isolation Kit** # 130-075-101 or # 130-075-201). When working with RNA, always use RNase inhibitors to prevent degradation of RNA.

2.1 Generation of the biotinylated cDNA pool (subtractor cDNA)

The generation of biotinylated cDNA from mRNA is accomplished by using 5'-biotinylated Oligo(dT) primer in a Reverse Transcription reaction. We recommend HPLC-purified primer with about 15-18 (dT). Remove the mRNA from the mRNA/cDNA hybrids by using RNase H. Inactivate RNase H prior hybridization by phenol extraction.

2.2 Hybridization

1. Mix the target mRNA and the subtractor DNA in about 100 µl of **Hybridization Buffer**. Prehybridization (e.g. with salmon sperm DNA) is not necessary. Heat denature for 10 minutes at 70 °C and quickly chill on ice for further 5 minutes.
2. Hybridize at 68 °C overnight. To reduce the stringency, you may lower the temperature to 42°C or even to room temperature. The salt concentration of the hybridization buffer likewise affects the hybridization reaction: the lower the salt concentration, the higher the stringency. Suitable hybridization buffers are 0.1-2x SSC, 0.1-2x SSPE, 50 mM Tris-acetate pH 7.5 + 20-300 mM NaCl, and others.

▲ **Note:** Do not add denaturants like formamide as they may interfere with µMACS Streptavidin MicroBead performance.

2.3 Magnetic labeling

1. After the hybridization, place the reaction mixture on ice and add **µMACS Streptavidin MicroBeads**. 100 µl of the µMACS Streptavidin Microbeads bind up to 100 pmol of biotinylated oligonucleotides. For optimum results, the dilution of the µMACS Streptavidin Microbeads should be no more than 1:10.
2. Incubate for 5 minutes. If the temperature during capturing is lower than room temperature we recommend prolonging the capture time to up to 15 minutes.

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2.4 Magnetic separation

1. Place a µ Column in the magnetic field of the µMACS separator.
2. Prepare the column by rinsing with 100 µl of **Equilibration Buffer** for nucleic acid applications and with 2x100 µl of **Hybridization Buffer**.
3. Apply the hybridization mixture to the column and let it pass through. The magnetically labeled mRNA/cDNA is retained on the column.
4. Wash with 5 x 100 µl of **Hybridization Buffer**, reducing the salt concentration successively (e.g. 2x, 1x, 0.5x, 0.25x, 0.1x SSC).
5. Collect the flow-through and all wash fractions.
6. The procedure may be repeated once or twice to increase the specificity. To reduce the volume, the eluate may be precipitated or concentrated by using a Speed-Vac®.
7. To analyze the eluted mRNA, it is recommended to transcribe it to cDNA with Reverse Transcriptase.

2.5 Appendix

Estimation of hybridization temperatures

The following formula is helpful to estimate the melting temperature of DNA/RNA hybrids to optimize the hybridization conditions:

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

where $[Na^+]$ represents the concentration of Na^+ ions in mol/l, % GC is the percentage of GC in the duplex, and n is the length in bp of the hybridizing part.