MACS molecular

Isolation of specific tRNA molecules

µMACS Streptavidin Kit Order No. 130-074-101

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1. Background

tRNA molecules play a major role in translation. The absence or presence of low and high abundant tRNA molecules is one of the most important factors which influence the translation rate and differs from species to species or from one cell line to another. To compare or identify the tRNA repertoire it is generally necessary to isolate the corresponding molecules via complex formation with DNA sequences.
It is known that a spectrum of agents, like colicin D, have the potential to cleave specific tRNA molecules, leading to cell death. In addition, the role of modifications of some tRNA species like methylation of 2'-OH groups is unclear. To answer open questions in these fields, the isolation of natural occurring and mutated tRNA molecules is often a prerequisite.

1.1 Reagent and instrument requirements

- **Buffers:**
  The DNA-tRNA binding can optionally be performed in the following buffer system. The composition of the buffers – especially with respect to pH, ionic strength, and the presence or absence of $MgCl_2$ – should be determined to optimize the binding of the tRNA of interest to the capture DNA.

  Resuspension Buffer: 0.3 M sodium acetate pH 4.5, 10 mM EDTA.

  Annealing Buffer (10X): 200 mM Tris/HCl pH 7.5, 50 mM $MgCl_2$, 25 mM spermidine, 1 mM DTT, 4 % Triton X-100.

  Binding/Wash Buffer (5X): 50 mM Tris/HCl pH 7.5, 5 mM EDTA, 2.5 M NaCl.

  Elution Buffer: 10 mM Tris/HCl pH 7.5, 1 mM EDTA.
- **µMACS™ Separator** (# 130-042-602)
- **MACS® MultiStand** (# 130-042-303)
- **µ Columns** (# 130-042-701)
- **µMACS Streptavidin Kit** (# 130-074-101

2. Protocol

2.1 Generation of the biotinylated capture DNA

To bind specific tRNA molecules, a corresponding single stranded DNA oligonucleotide must be synthesized, which is complementary to a particular segment of the tRNA. The length of the oligonucleotide should comprise 30-45 bases. The oligonucleotide must contain 1-2 biotin residues (5' and/or 3') and should be HPLC purified.

Biotinylated known single stranded DNA sequences are commercially available.

2.2 Enrichment of tRNA from cells

General outline for the isolation of the tRNA fraction from E.coli (adapted from Varshney et al. (1991) J. Biol. Chem. 266 (36), 24712-24718).

1. Pellet the cells (from 4 ml of medium) by centrifugation for 5 minutes at 300xg, 4°C. Resuspend the pellet in 300 µl of ice cold **Resuspension Buffer**.
2. Perform a **direct phenol extraction**: Add 300 µl of cold acid phenol (or acid phenol:chlorophorm 5:1, pH 4.5) to the cells and vortex for 30, 60 and 60 seconds with 60 seconds intervals between the steps.
3. Centrifuge for 10 minutes at 15,000xg, 4 °C.
4. Transfer the aqueous phase to a new tube containing 300 µl of cold acid phenol.
5. Vortex for 60 seconds, and centrifuge for 10 minutes at 15,000xg, 4°C.
6. Transfer the aqueous layer to a new tube containing 300 µl of cold acid phenol, mixed with 2.5 volumes (approx. 3.6 ml) of ethanol.
7. Incubate for 1-2 hours on ice.
8. Centrifuge for 15 minutes at 15,000xg, 4°C; this recovers the total of nucleic acids. Discard the supernatant.
9. Dissolve the pellet in 300 µl of cold 10 mM sodium acetate pH 4.5, mixed with 30 µl (1/10 volume) of 8 M LiCl.
10. Centrifuge for 15 minutes at 15,000xg, 4°C. The tRNA remains in the supernatant.
11. Store the supernatant in aliquots at -70 °C.
12. Check the quality by measuring the absorbance at 260/280 nm and by agarose gel electrophoresis. We recommend staining with SYBR® green II.

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2.3 Binding of specific tRNA molecules to the biotinylated capture DNA

1. Mix tRNA molecules with 1 µg of the biotinylated capture DNA and PEG-400 (final concentration 40 % (v/v)) in 1X **Annealing Buffer**. Keep the final volume as small as possible.
2. Incubate the mixture at 90°C for 2-3 minutes. Quickly cool down to 45°C (Thermocycler) and incubate overnight at 45°C.

▲ **Note:** The annealing temperature must be optimized according to the individual reaction mixture. The composition of the Annealing Buffer (e.g. salt concentration) can be varied for highly specific hybridization conditions.

2.4 Magnetic labeling of the DNA-tRNA complex

1. Preheat 5X **Binding/Wash Buffer** to 45 °C (or the corresponding optimized annealing temperature) and add to the binding mixture to a final concentration of 1X. The final volume should be between 400-900 µl.

▲ **Note:** In some cases, a final concentration of 1 M NaCl may be required for a stable DNA-tRNA complex formation.

2. Add 100 µl of **µMACS Streptavidin MicroBeads** and incubate for 5 minutes at 45°C. For optimum results, the dilution of the µMACS Streptavidin Microbeads should be no more than 1:10.

2.5 Magnetic separation

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 column, followed by washing with 2x100 µl of **1X Binding/Wash Buffer**.

▲ **Note:** In some cases, the **Binding/Wash Buffer** may require a final concentration of 1 M NaCl for a stable DNA-tRNA complex formation.

3. Apply the mixture containing magnetically labeled DNA-tRNA complexes to the µ Column and let it pass through. The magnetically labeled DNA-tRNA complexes are retained in the column.
4. Rinse the column with 4x100 µl of **Binding/Washing Buffer**.

▲ **Note:** The composition of the **Binding/Washing Buffer** may be adjusted (e.g. salt concentration) to achieve optimal washing conditions.

5. Preheat the **Elution Buffer** to 80 °C.
6. Elute the tRNA from the µ Column by adding 150-200 µl of hot **Elution Buffer**, while the column remains in the magnetic field. If the eluate should be more concentrated, collect the second to forth drop (the first drop usually does not contain any tRNA).

2.6 Analysis

The isolated tRNA may be analyzed by electrophoresis on acid urea gels and by Northern Blot Hybridization.

1. Mix tRNA with sample buffer (0.1 M sodium acetate (pH 5.0), 8 M urea, 0.05 % bromphenol blue, and 0.05% xylene cyanol) and fractionate on a 6.5% polyacrylamide gel containing 8 M urea in 0.1 M sodium acetate buffer (pH 5.0). Run at 12V/cm until the bromphenol blue dye reaches the bottom of the gel.
2. Blot the portion of the gel between the xylene cyanol and bromphenol blue dyes, which contains the tRNAs of interest, onto a Nytran membrane (Schleicher & Schuell) at 20 V for 90 minutes with 40 mM Tris-acetate, 2 mM EDTA (pH 8.1) as transfer buffer. Rinse the membrane briefly with 120 mM Tris/HCl pH 8, 0.6 M NaCl, 4.8 mM EDTA. UV-crosslink (120 mJ/cm^2), and bake at 80°C for 1-2 hours.
3. For the detection of tRNAs, use a 5‘-^{32}P-labeled oligonucleotide probe (10^6 cpm/ml) complementary to the corresponding tRNA.
4. Prehybridize the membrane at 42°C for 4 hours in a solution consisting of 120 mM Tris/HCl pH 8, 0.6 M NaCl, 4.8 mM EDTA, 250 pg/ml sheared and denatured salmon sperm DNA, 0.1 % SDS, and 10X Denhardt‘s solution (1X Denhardt‘s solution = 0.02 % BSA fraction V, 0.02 % polyvinylpyrrolidone 40, and 0.02 % Ficoll™).
5. Hybridize at 42 °C overnight in the same solution in the presence of the 5‘-^{32}P-labeled oligonucleotide probes.
6. Afterwards, wash membrane four times for 30 minutes each at room temperature with 90 mM Tris/HCl pH 8, 0.45 M NaCl, 3.6 mM EDTA, and 0.1% SDS, and expose to autoradiograph (x-ray) film.