mRNA Isolation Kit

for the isolation of poly(A)$^+$ RNA (at least 70 μg)

Cat. No. 11 741 985 001

Principle	The poly(A)$^+$ tail of mRNA hybridizes to a biotin-labeled oligo(dT) probe. Streptavidin-coated magnetic particles capture the biotinylated dT-A hybrids. A magnetic particle separator collects the magnetic particles, and washes remove contaminants. Then, water elutes the mRNA from the particles.
Starting material	Research samples may contain up to: • 2.5 mg total RNA • 10^8 cultured cells • 1 g tissue
Application	Preparation of highly purified poly(A)$^+$ RNA, which may be used for RT-PCR, cDNA synthesis, Northern blotting, Northern ELISA, RNase protection assays, *in vitro* translation, etc.
Time required	• Total time: approx. 30 min (starting from total RNA) • Hands-on time: approx. 15 min (starting from total RNA)
Results	• Yield: Variable, depending upon starting material (See Part IV of "How to use the kit" in this article.) • Purity: Isolated mRNA, free of DNA and other RNAs
Benefits	• **Saves time**, because the kit can isolate mRNA directly from cell lysates and tissue homogenates; no isolation of total RNA required • **Accommodates a wide range of samples**, and allows both small and large scale preparations of mRNA • **Increases lab safety**, because the kit does not use hazardous organic solvents

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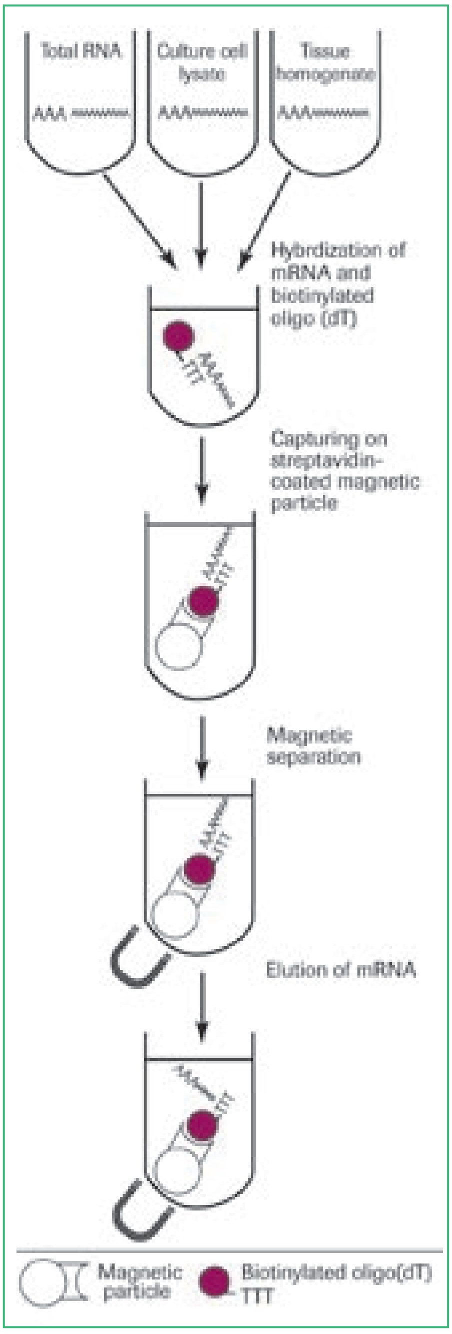
How to use the kit

I. Flow diagram



II. Kit contents

- Lysis Buffer containing lithium dodecyl sulfate (100 ml)
- Streptavidin-coated Magnetic Particles (1.7 ml)
- Oligo(dT)$_{20}$, biotin-labeled, ready-to-use (66 µl)
- Wash Buffer (50 ml)
- Double dist. Water, nuclease-free (4 ml)
- Storage Buffer (7 ml)

III. Additional materials needed

- Magnetic Particle Separator
- Sterile tubes and cups
- Pipettes with sterile, disposable pipette tips
- Syringe fitted with a 21-gauge needle (for tissue and cultured cells only)
- PBS (for preparing cultured cells only)
- Mortar, pestle, liquid nitrogen (for preparing tissue samples only)

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IV. Typical mRNA content of different samples

Starting material	Total RNA (µg)	mRNA (µg)
Cultured cells (10^7 cells)	30 – 500	0.3 - 25
Tissue (100 mg):		
Mouse brain	200	7
Mouse liver	700	14
Mouse lung	130	10

V. Protocol for isolating mRNA from 500 µg total RNA, 2 x 10^7 cultured cells, or 200 mg tissue

General notes:

- Make sure that all material which contacts the RNA is free of contaminating RNases. For decontamination of equipment, see Appendix (page 219).
- The procedures below apply only to the isolation of mRNA from the specified amounts of starting material (500 µg total RNA, 2 x 10^7 cultured cells, or 200 mg tissue). To isolate mRNA from different amounts or volumes of sample, you must alter the amounts of reagents used in the procedures below. For details, see the package insert provided with the mRNA Isolation Kit.

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1. Prepare the sample:
   - Total RNA: Dilute 500 µg total RNA (up to 200 µl) with Lysis Buffer (final total volume, 400 µl). Incubate for 2 min at 65°C.

     ! *Do not dilute the Lysis Buffer more than twofold.*

▶▶▶

- Cultured cells: Wash cells (2 x 10^7) twice with ice-cold phosphate-buffered saline (PBS). Add 3 ml Lysis Buffer to the cell pellet. Shear DNA mechanically by passing the sample 6 x through a 21-gauge needle.
- Tissue: Snap freeze 200 mg of tissue. Grind frozen tissue to a homogeneous powder in a pre-cooled mortar. Chill 3 ml Lysis Buffer to ≤0°C in a sodium chloride-ice water bath, then add the frozen powder to the chilled Lysis Buffer. Homogenize the powder suspension by passing it 4 x through a 21-gauge needle. Centrifuge the suspension at 11,000 x *g* for 30 s. Use only the supernatant for the capture procedure.

  Perform all steps in the capture procedure below at a temperature between 0°C and –4°C.

2. Prepare the Streptavidin-coated Magnetic Particles:
   - Resuspend Streptavidin-coated Magnetic Particles thoroughly and pipette 300 µl Streptavidin-coated Magnetic Particles into a fresh cup or tube.
   - Immobilize the Streptavidin-coated Magnetic Particles on the side of the container with a Magnetic Particle Separator.
   - Remove the storage buffer from the Streptavidin-coated Magnetic Particles.
   - Resuspend the Streptavidin-coated Magnetic Particles in 500 µl Lysis Buffer.
   - Again immobilize the Streptavidin-coated Magnetic Particles with the magnetic separator and remove all the Lysis Buffer.

   Never let the Streptavidin-coated Magnetic Particles dry out.

3. Hybridize the mRNA to the Biotin-labeled Oligo$(dT)_{20}$:
   - Add 3 µl Biotin-labeled Oligo$(dT)_{20}$ to the sample (from Step 1)
   - Mix sample and Oligo$(dT)_{20}$ well to form the hybridization mix.

4. Immobilize the biotinylated dT-A hybrids with Streptavidin-coated Magnetic Particles:
   - Add hybridization mix to the tube containing the prepared Streptavidin-coated Magnetic Particles (from Step 2).
   - Resuspend Streptavidin-coated Magnetic Particles in the hybridization mix.
   - Incubate for:
     - EITHER 5 min at 37°C (if you started with total RNA or cultured cells).
     - OR 5 min at 0°C (if you started with tissue).

5. Separate the Streptavidin-coated Magnetic Particles from the fluid with a Magnetic Particle Separator.

   This step takes about 3 min.

6. Wash the Streptavidin-coated Magnetic Particles 3 times. For each wash:
   - Add 500 µl Wash Buffer to the tube.
   - Resuspend the Streptavidin-coated Magnetic Particles in the Wash Buffer.
   - Separate the Streptavidin-coated Magnetic Particles from the buffer with a Magnetic Particle Separator.
   - Remove all Wash Buffer and discard.

7. Elute the mRNA:
   - Add 50 µl redistilled water to the Streptavidin-coated Magnetic Particles.
   - Resuspend the Streptavidin-coated Magnetic Particles in the redistilled water.
   - Incubate for 2 min at 65°C.
   - Separate Streptavidin-coated Magnetic Particles from the eluate with a Magnetic Particle Separator.

8. Transfer the supernatant (containing the mRNA) to a fresh RNase-free tube.

   To quantitate the mRNA spectrophotometrically, assume that 1 A_{260} unit corresponds to a concentration of 40 µg/ml.

VI. Troubleshooting the mRNA Isolation protocol

If you get...	Then, the cause may be...	And you should...
Low yield or no yield	Insufficient homogenization of sample material	▶ Eliminate all visible clumps in lysate (Step 1) by homogenization, etc. ❗ *mRNA trapped inside sample clumps cannot be isolated by this procedure.*
	Too much sample material per volume of Lysis Buffer (lysate viscosity too high)	▶ Use no more than the recommended amount of starting material. ❗ *The viscosity of the lysate is critical to successful mRNA isolation.*
	Elution temperature too low	▶ Incubate suspension in double dist. water for 2 min at 65°C (Step 7).
	Elution volume too low	▶ Repeat elution.
	Improper preparation and reuse of magnetic particles	▶ Follow instructions in the package insert if you intend to reuse the Streptavidin-coated Magnetic Particles. ❗ *Do not regenerate Streptavidin-coated Magnetic Particles with alkali.*
	Wrong lysis buffer or lysis buffer with too much guanidine thiocyanate (GTC)	▶ Use only the Lysis Buffer provided in the kit. ▶ If you use a lysis buffer containing GTC, follow the guidelines in the package insert for GTC concentration and hybridization temperature.
	RNase contamination in buffers	▶ Check all buffers for RNase contamination. ▶ Follow published procedures (*e.g.*, Farrel, 1983) for RNase decontamination.
Degraded mRNA	RNase contamination in buffers	▶ Check all buffers for RNase contamination. ▶ Follow published procedures (*e.g.*, Farrel, 1983) for RNase decontamination.
	Sample material processed too slowly or at a too high temperature	▶ Follow the sample preparation guidelines in Step 1
rRNA or DNA contamination	Too much sample material per volume of lysis buffer (lysate viscosity too high)	▶ Use no more than the recommended amount of starting material. ▶ Use the isolated mRNA as starting material and repeat the isolation protocol.

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Typical results with the kit

Experiment 1

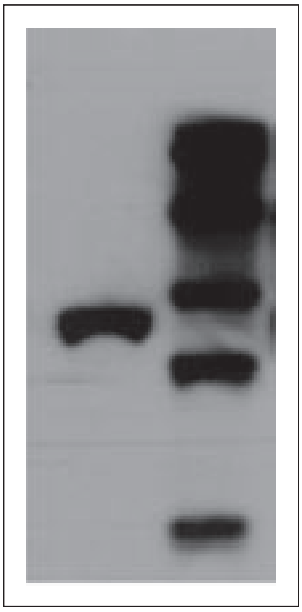

Figure 49: Northern blot with liver mRNA prepared with the mRNA Isolation Kit. mRNA was isolated from liver tissue according to the above protocol. The mRNA was separated electrophoretically, transferred to a membrane by Northern blotting, and analyzed with a DIG-labeled actin cDNA probe. The hybrids were visualized with an enzyme-labeled anti-DIG antibody and a chemiluminescent enzyme substrate.

Result: A single, strong actin band was visible in the mRNA (left lane). No degradation products were visible.

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Experiment 2

Detection of Angiotensin Converting Enzyme (ACE) mRNA in chicken eye.

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Background: In the last decade, research has increasingly focused on the role of peptides in the physiology of the central nervous system and in the retina. Specifically, attention focused on the renin-angiotensin system (RAS), in which angiotensinogen is cleaved by renin and angiotensin-converting enzyme (ACE) to produce the active peptide angiotensin II (Oparil and Haber, 1974).

Originally, the RAS was thought to be solely a circulatory endocrine system. However, increasing evidence suggests that an RAS is expressed locally in various tissues. In these tissues, the RAS is believed to act as a functionally independent paracrine/autocrine system (Unger et al., 1991).

By using RT-PCR, we have been able to detect ACE-specific mRNA in several tissues from chicken eye. To obtain enough mRNA template from these neuronal tissues, we used the mRNA Isolation Kit.

Methods: *Preparation of mRNA from chicken eye*. The eyes of each chicken (*Gallus domesticus, 12 days old*) were dissected immediately after enucleation by hemisection along the ora serrata. The optic nerve head, pecten, retina, choroid, and the iris/ciliary body were carefully prepared and frozen in liquid nitrogen. Tissues were homogenized and mRNA isolated with the mRNA Isolation Kit, according to the protocol given in the kit package insert.

Reverse transcription. Isolated mRNA samples were treated with RNase-free DNase, extracted with phenol, and precipitated with ethanol, according to standard procedures. The treated mRNA samples were reverse transcribed with the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV).

ACE-specific PCR. Optimal PCR conditions were determined with the PCR Optimization Kit. The entire product (20 µl) of the reverse transcription reaction was amplified in a 100 µl reaction mix containing 10 mM Tris HCl (pH 8.8), 75 mM KCl, 1.5 mM $MgCl_2$, 3% dimethylsulfoxide, 10 mM dNTPs, 15 pmol of each ACE-specific primer, and 2.5 units Taq polymerase. The sequences of the ACE-specific primers were deduced from EMBL; AC: L40175 (Esther et al., 1994):

5'-ACAACAAGACCAATGAGGTGC-3' (sense primer)

5'-CCTTCCAGGCAAAGAGGAG-3' (antisense primer)
After an initial denaturation step (5 min, 95°C), samples were subjected to 30 PCR cycles [1 cycle = denaturation (45 s, 94°C), annealing (1 min, 60°C), and elongation (1 min, 72°C)]. The final extension was 10 min at 72°C.

Results and conclusions: We obtained at least 3 µg pure mRNA per chicken retina (i.e., 8.7 – 9.3 µg mRNA/100 mg tissue) in <1 h with the mRNA Isolation Kit. The yield was highly reproducible (data not shown).

Starting from the isolated mRNA, the RT-PCR produced an ACE-specific 525 bp-amplicon (Figure 50). The specificity of the amplified sequence was tested by digestion of the amplicon with Pvu II. As expected, the amplicon was cleaved into two fragments (137 bp and 388 bp) (Figure 50).

These results indicate that ACE gene expression occurs in the retina, choroid, pecten, optic nerve and iris/ciliary body of the chicken eye. The results strongly support the hypothesis that ACE-specific mRNA is localized in retinal neurons or glia.

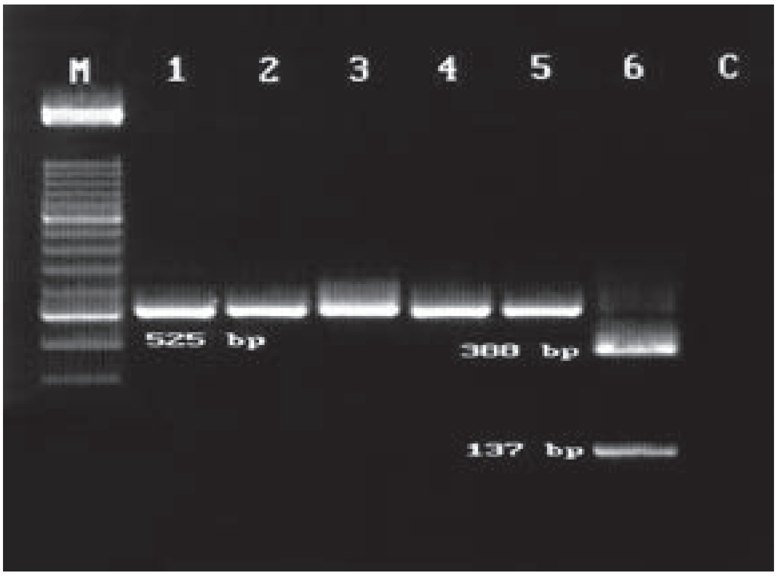

Figure 50: Detection of ACE-specific mRNA in chicken eye tissue by RT-PCR. mRNA was isolated from chicken eye tissue with the mRNA Isolation Kit, as described in the text. The mRNA was amplified by RT-PCR, using primers derived from the gene for angiotensin converting enzyme (ACE). Amplified DNA products were run on a 1.5% agarose gel and stained with ethidium bromide.

Lane 1: Amplicon from retina
Lane 2: Amplicon from choroid
Lane 3: Amplicon from pecten
Lane 4: Amplicon from iris/ciliary body
Lane 5: Amplicon from optic nerve
Lane 6: Amplicon after Pvu II digestion
Lane M: DNA Molecular Weight Marker XIV (100 bp ladder)
Lane C: Negative control

Result: A single, strong 525 bp amplicon was present in all tissues. Pvu II split the amplicon into two fragments of 388 bp and 137 bp.

References

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