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Print Protocol

Print Changes to Previous Version

mRNA Capture Kit

Version 7.0
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Cat. No. 11 787 896 001
192 reactions

Kit for capturing of poly (A^+) RNA from total RNA, cell lysates and tissue homogenates in PCR tubes

Store at +2 to +8°C

www.roche-applied-science.com

Contents

Kit contents

1. Lysis buffer, ready-to-use solution containing less than 2% lithium dodecylsulfate	1 × 50 ml	translucent bottle
2. Oligo(dT)$_{20}$, biotin-labeled, 20 × stock solution containing 5 nmol of oligonucleotide	1 × 50 µl	translucent cup red cap
3. Nuclease-free redist. water	1 × 1 ml	translucent cup translucent cap
4. Washing buffer, ready-to-use solution	5 × 100 ml	translucent bottles
5. Streptavidin-coated PCR tubes (strips)	24 × 8	
6. Caps for the PCR tubes (strips)	24 × 8	

Stability and storage

The kit is stable until the expiry date given on the kit label, if stored at +2 to +8°C. For stability of working solutions, see section 4.2.1.

Specifications of the streptavidin-coated PCR tubes

Streptavidin-coated area: 200 µl
Total binding capacity for biotin: 15 ng/tube (200 µl)
Coating heterogeneity between individual tubes: SD < 5%.
The tubes are free of ribonuclease activity, according to the current quality control procedures.
Note: The binding capacity as given under specifications is identical to the number of binding sites present.

Advantages of the mRNA Capture Kit

- **Fast and easy**: one tube reaction
- **Flexible:** Adaptable from small to high numbers of samples and semi-automatic procedures possible.
- **Efficient:** Avoid loss of sample mRNA
- **Economic**: Cost-effective procedure.

1. Introduction

Usually RT-PCR (reverse transcription polymerase chain reaction) is performed with purified total RNA or mRNA as starting material because cell lysates or tissue homogenates often contain a number of polymerase inhibitors. To combine the process of sample preparation and RT-PCR in a convenient and cost-effective way, we developed the mRNA Capture Kit. Cells, tissues or total RNA preparations are used as starting material. mRNA, which ends in a 3´-poly(A) tail, has to be released from the cells by a lysis step. The poly(A) residue of the mRNA serves then as a hybridization tag for biotin-labeled oligo(dT)$_{20}$. The oligo(dT) has two functions:

1. Capturing of poly(A$^+$) RNA in streptavidin-coated PCR tubes,
2. Priming of the reverse transcriptase reaction after washing off the lysate supernatant.

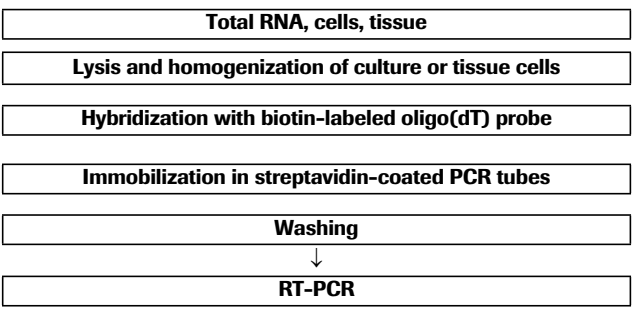
After immobilization the mRNA can be directly processed by addition of the reverse transcription mix. In the next step, the reverse

transcription mix is exchanged by a PCR mix. The PCR reaction can be done with or without labeled nucleotides (DIG-dUTP, fluorescein-dUTP) and the generated amplicon can be analyzed by conventional gel electrophoresis or by an ELISA-like procedure. Instead of a two step reaction using a reverse transcriptase and a thermostable DNA Polymerase sequentially a one-step procedure using Tth DNA polymerase* or a suitable enzyme combination can also be applied (3).

2. Application

Capturing of poly(A^+) RNA from total RNA, cells or tissues after lysis and homogenization and immobilization in streptavidin-coated PCR tubes. The immobilized mRNA can be used for qualitative or quantitative RT-PCR.

3. Test principle



4. Equipment and solutions

4.1 Additionally required equipment

PCR thermal cycler; mineral oil (if required for the particular thermal cycler used); pipettes; sterile aerosol-preventive pipette tips; sterile cups for preparing dilutions; syringe; 21 gauge needle; mortar, pestle and liquid nitrogen (if tissue is used as starting material); equipment for electrophoresis of DNA or other means for the analysis of PCR products, *e.g.*, PCR ELISA*.
Note: Ensure, that the material which gets in contact with RNA is free of contaminating RNases. Protocols for decontamination are described in the literature (1, 2).

4.2 Preparation and stability of the kit components

4.2.1 Working solutions

Solution 1: Lysis buffer (bottle 1)
Ready-to-use solution. The solution is stable until the expiry date given on the kit label, if stored at +2 to +8°C.

Solution 2a: Oligo(dT)$_{20}$, biotin-labeled (bottle 2)
The solution is stable until the expiry date given on the kit label, if stored at +2 to +8°C.

Solution 2b: Oligo(dT)$_{20}$, biotin-labeled; working solution
Dilute an appropriate volume of solution 2a with nuclease-free redist. water (solution 3) (1 : 20). 4 µl of diluted oligo(dT)$_{20}$ are needed for one reaction. The working solution should preferably be prepared freshly. It may also be stored at -15 to -20°C, however, repeated freezing/thawing should be avoided.

Solution 3: Nuclease-free redist. water (bottle 3)
Nuclease-free redist. water is required for the preparation of the oligo(dT)$_{20}$ working solution (solution 2b). Avoid contamination of the nuclease-free redist. water with nucleases or microorganisms.

Solution 4: Washing buffer (bottle 4)
Ready-to-use solution. The solution is stable until the expiry date given on the kit label, if stored at +2 to +8°C.

4.2.2 Required solutions for RT-PCR

Reverse transcription (RT) mix; not provided with the kit
For reverse transcription of mRNA any suitable enzyme (*e.g.*, reverse transcriptase AMV*or M-MuLV*) can be used. Of course for a coupled RT-PCR step in one reaction mix, enzymes with both reverse transcriptase and thermostable polymerase activity (*e.g.*, Tth) can also be used. Most suppliers deliver the enzymes together with suitable buffers. If the customer would like to prepare his own mix, we recommend a composition as described by Sambrook et al. (2):

Volumes for preparation of 1 ml:

204.1 µl	10 mM Tris-HCl, pH 7.4,
51.0 µl	1 M Tris-HCl, pH 8.3 (42°C),
142.9 µl	1 M KCl,
40.8 µl	250 mM $MgCl_2$,
102.0 µl	dNTPs, 10 mM each,
40.8 µl	100 mM DTE,
20.0 µl	RNase inhibitor (40 U/µl)*,
398.4 µl	redist.water,
32.0 µl	AMV reverse transcriptase (25 U/µl)*.

The mix has always to be prepared freshly.

PCR mix; not provided with the kit
For amplification of cDNA any suitable enzyme or enzyme mix (*e.g.*, Taq DNA Polymerase*, Expand Long Template PCR System*) can be used. Most suppliers deliver the enzymes together with suitable buffers. We recommend to use the PCR Master* or the PCR Core Kit*. If the customer would like to prepare his own mix, a composition is described by Sambrook et al. (2).

4.2.3 Streptavidin-coated PCR tubes Use only the number of strips required for the particular experiment. Close the bag containing the remaining strips tightly. The coated tubes are stable until the expiry date given on the kit if stored at +2 to +8°C. Tubes which had not been loaded with solutions and "cycled dry" retain their biotin binding capacity.

5. Procedure

5.1 General considerations

Note: Decontaminate all equipment and solutions used for the work with RNA from RNases whenever possible. Laboratory glassware should be rendered RNase-free by baking at +180 °C for 8 hours. Plasticware should be treated with DMDC (dimethyldicarbonate). Sealed sterile plasticware can be used without prior RNase decontamination. Treat all solutions, which are not included in the kit and get in contact with RNA with DMDC whenever possible to destroy RNases. DEPC (diethylpyrocarbonate) may also be used for this purpose, but is a greater health hazard and more laborious to handle than DMDC. Tris buffer can not be decontaminated with DMDC. Tris buffer should be prepared by using DMDC treated water and autoclaving. For DMDC or DEPC treatment use 0.1% (v/v) solutions and incubate for 30 min at +15 to +25°C. Surplus DMDC or DEPC in the solutions is destroyed by autoclaving afterwards.
Wear disposable gloves to avoid RNase contamination of solutions and equipment by handling.
PCR is an ultra-sensitive method for amplification of DNA, even when present in a very low copy number. The mRNA captured in the PCR tube may be contaminated with minute amounts of DNA and lead to a positive signal after amplification even in the absence of specific cDNA. To avoid such false positive results we recommend the use of intron spanning primers for PCR. The control reaction may be performed by deleting the reverse transcriptase from the RT mix, which should not result in a PCR product.

5.2 Preparation of starting material

Total RNA: Dilute up to 40 µg of total RNA in 200 µl of lysis buffer.
Culture cells: Wash cells (up to 5×10^5) twice with ice-cold phosphate-buffered saline (PBS). Add 200 µl of lysis buffer to the cell pellet. Shear DNA mechanically by passing the sample six times through a 21 gauge needle.
Tissue: Grind up to 20 mg of snap-frozen tissue (liquid nitrogen) in a pre-cooled mortar to a homogeneous powder. Prevent thawing of the material. Add the frozen powder to 200 µl of the lysis buffer (pre-cooled to 0°C to –4°C, sodium chloride ice water bath). Homogenize by passing four times through a 21 gauge needle and spin down at 11 000 × g for 30 s afterwards. Separate the supernatant for the next steps. Perform the following hybridization, immobilization and washing step at 0°C to –4°C.

5.3 Hybridization of mRNA with the biotin-labeled oligo(dT)$_{20}$ probe

Add 4 µl of the biotin-labeled oligo(dT)$_{20}$ working solution (solution 2b) to the sample and incubate for 5 min at +2 to +8°C (tissue samples) or +37°C (culture cells, total RNA).

5.4 Immobilization of poly(A$^+$) RNA in streptavidin-coated PCR tubes

Add 50 µl of the mix to a streptavidin-coated PCR tube and incubate for 3 min at +2 to +8°C (tissue samples) or +37°C (culture cells, total RNA).

5.5 Washing

Remove mix from the tube and wash the tube three times using 250 µl of washing buffer (bottle 4). Be sure that the washing buffer is quantitatively removed after each washing step.
Do not wash too vigorously to avoid loosing mRNA.
The captured mRNA can now be used directly for reverse transcription and amplification in a RT-PCR assay.

6. Downstream applications

6.1 Synthesis of first strand cDNA

Add 50 µl of reverse transcription mix to the tube and incubate for the appropriate time at the appropriate temperature (dependending on the reverse transcriptase ; work according to the protocols recommended by the supplier). If the reverse transcription mix (described in this package insert) is used for the reverse transcription process, incubate for 120 min at +42°C.

6.2 Washing

Remove reverse transcription mix and wash once using 250 µl of washing buffer (bottle 4).

6.3 PCR reaction

Add a PCR mix to the PCR tube and start PCR process. A standard protocol which has to be optimized for the individual primers is: first cycle: 5 min at +94°C; 2 min at +50°C; 3 min at +72°C; subsequent cycles (up to 40 cycles): 1 min at +94°C; 2 min at +50°C; 3 min at +72°C; last cycle: 1 min at +94°C; 2 min at +50°C; 10 min at +72°C. If detection of the PCR product in an ELISA like format is intended, we recommend the use of the PCR ELISA (DIG-Labeling)* for the PCR process.

6.4 Detection of the PCR product

6.4.1 Electrophoresis and ethidium bromide staining

Work according to established protocols (2).

6.4.2 ELISA

If the PCR product has been DIG-labeled, it can be detected by using the PCR ELISA (DIG-Detection)*. Detailed protocols of the procedure are contained in the package insert.

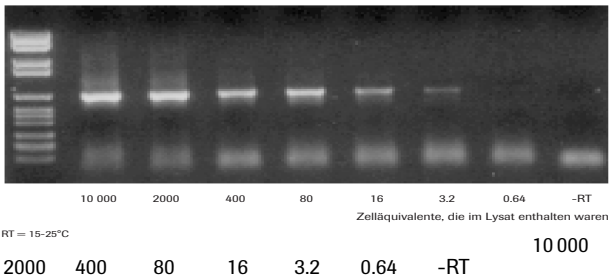


Fig. 1: Detection of ß-actin transcript in K562 cells. A cell lysate was prepared as described (section 5), diluted and hybridized with biotin-labeled oligo$(dT)_{20}$. RT-PCR was performed in two steps by using reverse transcriptase AMV and Taq DNA polymerase. Upper primer: CCA AGG CCA ACC GCG AGA AGA TGA C; lower primer: AGG GTA CAT GGT GGT GCC GCC AGA C; -RT: reverse transcription step without addition of reverse transcriptase (lysate corresponding to 10 000 cells).

7. Interpretation of the results

The presence of a PCR product after RT-PCR indicates that the corresponding mRNA was present in the sample. Quantification of the specific mRNA in the sample is also possible, if a suitable protocol for quantitative RT-PCR has been used.

8. Additional informations

Source	Total RNA (μg)/10^7 cells or 100 mg tissue	mRNA (μg)/10^7 cells or 100 mg tissue
Culture cells	30–500	0.3–25
Brain (mouse)	200	7
Liver (mouse)	700	14
Lung (mouse)	130	10

Tab. 1: RNA content of cells and tissues

9. References

1 Farrell, R. E.(1993) RNA Methodologies: A Laboratory Guide for Isolation and Characterization. Academic Press, New York.

2 Sambrook, J., Fritsch, E, F., & Maniatis, T. (1989): Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

3 PCR Applications Manual, available from Roche Molecular Biochemicals. ISBN 3-88630-195-8

*available from Roche Applied Science

9.1. Changes to previous version

Disclaimer of License deleted.
Editorial changes.

9.2 Regulatory Disclaimer

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9.3 Trademarks

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10. Quick reference protocols

10.1 Required solutions

Solution	Content	Volume per reaction	Used for (see: 10.2)
1	Lysis buffer (bottle 1); ready-to-use	200 µl	step 1
2a	Oligo$(dT)_{20}$, biotin-labeled (bottle 2); stock solution	0.2 µl	solution 2b
2b	Oligo$(dT)_{20}$, biotin-labeled; working solution	4.0 µl	step 2
3	Nuclease free redist. water (bottle 3)	3.8 µl	solution 2b
4	Washing buffer (bottle 4); ready-to-use	1.0 ml	steps 4, 6
5	Reverse transcription mix, not provided with the kit		step 5
6	PCR mix, not provided with the kit		step 7

10.2 Working procedure flow sheet

	Steps	Procedure	Volume	Time/ Temperature
1	Preparation of starting material a) Total RNA	Dilute up to 40 µg of RNA in lysis buffer.	200 µl	+2 to +8°C
	b) Cultured cells	Wash cells in PBS; add lysis buffer to a pellet of up to 5×10^5 cells and pass 6 × through a 21 gauge needle.	200 µl	+2 to +8°C
	c) Tissue	Grind up to 20 mg of frozen tissue on liquid nitrogen in a mortar to a homogeneous powder; add powder to the lysis buffer and pass 4 × through a 21 gauge needle; spin 30 s at 11 000 × g. Separate the supernatant for the next steps.	200 µl	0°C to –4°C
2	Hybridization with biotin-labeled oligo$(dT)_{20}$	Add biotin-labeled oligo$(dT)_{20}$ working solution (solution 2b) and incubate.	4 µl	5 min at +37°C or +2 to +8°C (tissue)
3	Immobilization	Transfer hybridization mix into streptavidin-coated PCR tube and incubate.	50 µl	3 min at +37°C or +2 to +8°C (tissue)
4	Washing	Remove mix from the tube and wash 3 times with washing buffer (solution 4).	250 µl	+15 to +25°C

Continued on next page

10.2 Working procedure flow sheet, Continued

	Steps	Procedure	Vol.	Time/Temp.
5	Synthesis of first strand cDNA	Add reverse transcriptase mix and incubate.	50 µl	120 min at +42°C
6	Washing	Remove mix from the tube and wash once with washing buffer (solution 4).	250 µl	1+15 to +25°C
7	PCR reaction	Add PCR mix and start PCR programm[1].		**Cycle 1:** 5 min, +94°C 2 min, +50°C 3 min, +72°C **Cycles 2 up to 40:** 1 min, +94°C 2 min, +50°C 3 min, +72°C **Last cycle:** 1 min, +94°C 2 min, +50°C 10 min, +72°C
8	Detection	According to established protocols: electrophoresis and staining (see ref. 2), PCR ELISA[2]		

[1] The program described here is only a first choice protocol. However, for optimal results cycling parameters have to be adjusted to your specific application. Depending on the type of thermal cycler, it may be necessary to overlay with mineral oil.

[2] Detection of the amplicon by PCR ELISA necessitates its labeling with DIG. This is easily done by the PCR ELISA (DIG-Labeling.)

11. Ordering Information

Product	Pack size	Cat. Nos.
Oligo(dT)$_{20}$ probe, biotin-labeled	2 nmol	1 1741 764 001
Streptavidin-coated PCR tubes	192 tubes	11 741 772 001
PCR Master	100 reactions	11 636 103 001
PCR Nucleotide Mix	100 reactions	11 581 295 001
PCR Core Kit	100 reactions	11 578 553 001
PCR ELISA (DIG-Labeling)	50 reactions	11 636 120 001
PCR ELISA (DIG-Detection)	192 reactions	11 636 111 001
Reverse Transcriptase AMV	500 units	11 495 062 001
Reverse Transcriptase M-MuLV	500 units	11 062 603 001
Taq DNA Polymerase	100 units	11 146 165 001
Tth DNA Polymerase	100 units	11 480 014 001
Expand Long Template PCR System	100 units 2 x 250 units	11 681 834 001 11 681 842 001
cDNA Synthesis Kit	Kit for 25 µg RNA	11 117 831 001
First Strand DNA Synthesis Kit for RT-PCR	30 reactions	11 483 188 001
Transcriptor First Strand cDNA Synthesis Kit	50 reactions	04 379 012 001

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