EURx® MOLECULAR BIOLOGY PRODUCTS

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Version 2.1
October 2019

GeneMATRIX FFPE RNA Purification Kit

Kit for purification of total RNA from formalin-fixed, paraffin-embedded (FFPE) tissue sections.

Cat. no. E3593

EURx Ltd. 80-297 Gdansk Poland
ul. Przyrodnikow 3, NIP 957-07-05-191
KRS 0000202039, www.eurx.com.pl
orders: email: orders@eurx.com.pl
tel. +48 58 524 06 97, fax +48 58 341 74 23

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Content	25 preps E3593-01	100 preps E3593-02	Storage/Stability
Lyse ALL	6 ml	24 ml	15-25°C
RL	18 ml	72 ml	15-25°C
Proteinase K (20 mg/ml)	0.45 ml	1.8 ml	–20°C
Wash RNA	39 ml	156 ml	15-25°C
DNR II	0.6 ml	2.4 ml	2-8°C
RNase-free water	3.6 ml	15 ml	15-25°C
DNase I	0.06 ml	0.24 ml	–20°C
Homogenization Columns	25	2 x 50	15-25°C
RNA Binding Columns	25	2 x 50	15-25°C
Protocol	1	1	

RNA Isolation from FFPE Tissue Samples: A Brief Outline of the Protocol

- **Paraffin Removal.** In a first step, paraffin is removed from the tissue sample. Two suitable procedures are proposed, (1) Xylene treatment and (2) Heptane and Methanol treatment. It is possible to choose alternate methods for paraffin removal.
- **Lysis and Homogenization.** Resuspension and lysis of the pelleted sample is followed by Proteinase K digestion. RNA modifications by fixative agents such as formaldehyde are partially reversed during heat treatment. The processed sample lysate is transferred to a homogenization spin-column. During homogenization spin-column passage, both DNA and RNA remain in solution and are collected with the flow-through. DNA is removed by DNase I treatment.
- **RNA Binding and Washing.** Remaining traces of DNA in the RNA-containing flow-through are removed in a DNase I digestion step. In turn, the flow-through is applied to the RNA binding spin-column. RNA molecules get adsorbed to the RNA-binding column matrix with high selectivity. Contaminants — even chemically similar compounds — interact only weakly with the matrix and are efficiently removed after two washing steps.
- **Elution.** High-quality RNA is then eluted in RNase-free water.
- **Cutoff.** The kit has no cutoff size, thus enabling the isolation of small to large sized RNA.

Equipment and reagents to be supplied by the experimenter

1. For deparaffinization of FFPE tissue sections: xylen or heptane and methanol.
2. Ethanol [96–100% v/v], microcentrifuge, disposable gloves, sterile RNase-free pipet tips, sterile RNase-free 1.5–2 ml tubes, vortexer, heating block capable of incubation at 50–80°C. Optional: equipment for sample disruption and homogenization.

Introductory Notes

NOTE 1 · Kit Specification. This kit is specially designed for isolation of total RNA together with small RNA molecules (15–30 nucleotides) from formalin-fixed, paraffin-embedded (FFPE) tissue sections.

NOTE 2 · Maximum Sample Volume and Column Binding Capacity. One mini column allows purification of RNA from a maximum of up to 2–4 sections, 5–20 µm thickness each. Sum of thickness should not be more than 40 µm. The total RNA binding capacity is 125 µg per spin-column. The maximum volume of the column reservoir is 650 µl.

NOTE 3 · Kit Compounds Storage. Keep all solutions tightly closed to avoid evaporation, resulting in com-ponents concentration changes. Store all components of the kit at 15–25°C with the exception of DNRII, DNase I and Proteinase K. DNase I and Proteinase K should be kept at -20°C. DNRII should be kept at 2–8°C.

NOTE 4 · Try Using Non-Autoclaved Plasticware. Autoclaved plastic ware is sterile, but may not be com-pletely RNase- and DNA-free. Neither RNases nor DNA do completely disintegrate at 120°C. Autoclave-borne aerosols are known to transport traces of RNase and of DNA into autoclaved liquids and onto the surface of plastic ware. In particular, RNases are known as tough enzymes with the capability for partial refolding at room temperature, even upon autoclave treatment. Do not autoclave mission-critical liquids and plastic ware, especially if autoclaves were previously used for preparation of bacterial agar, for media or for sterilization of lab waste. Use of two separate autoclaves, one reserved for defined buffers and plastic ware only, and one for sterilizing complex media and lab waste, is highly recommended. With high quality plasticware, autoclaving is not required: Modern high quality plastic reaction tubes are produced without any human interference, are per default DNA and RNase free, and, when stored clean and dust free, should best be used without any autoclave treatment. For convenient storage of plastic tubes at the lab bench, dry-sterilize a set of glass beakers (common preserving jars are perfectly suited for this purpose) and, following cooling, aliquot a freshly opened bag of high quality plastic reaction tubes into each of the sterilized beakers. Close all beakers with the accompanying lid. Always use a clean forceps (not gloved hands or even bare hands) for picking individual plastic reaction bottles during daily lab routine work.

NOTE 5 · General FFPE RNA Quality Considerations. The method of archiving tissue in formaldehyde and paraffine adversely affects the quality of nucleic acids. DNA/RNA molecules are usually heavily fragmented, cross-linked, and chemically modified by formaldehyde. The degree of fragmentation depends on the type and age of the sample as well as on the conditions for fixation, embedding, and storage of the sample. The purification procedure is optimized to reverse as much formaldehyde modifications as possible, without further RNA degradation, but nucleic acids purified from FFPE samples should not be used in downstream applications requiring full-length RNA. The kit is well suited for the recovery of usable RNA fragments in applications such as RT-PCR.

NOTE 6 · Use high quality reverse transcriptases when working with impaired RNA, such as FFPE tissue recovered RNA samples. The following reverse transcriptases were tested for downstream compatibility with this kit and are known to work very well: For maximum sensitivity and specificity, choose a two-step RT-PCR approach with AMV Reverse Transcriptase Native (Cat. No. E1372) or with the NG dART RT kit (Cat. No. E0801). For optimized reproducibility with respect to quantitative analyses, use a one-step RT-PCR approach with the One Step RT Kit (Cat. No. E0803) or with the SG OneStep RT-qPCR Master Mix (Cat. No. E0810).

NOTE 7 · DNase I is included with the kit and does not need to be purchased separately.

Protocol

Part I Removal of paraffin

The first processing step in the case of formalin-fixed, paraffin-embedded (FFPE) tissue sections is to dissolve and remove the paraffin. Two methods for paraffin dissolution are described below. When using alternate methods for paraffin removal, ensure that the resulting pellet is completely dry and free of organic solvent traces, before continuing with further sample processing according to Part II of the Protocol.

Xylene

1. Add 1 ml of xylene to the sample. Vortex for 15 sec. Centrifuge the sample for 2 min at room temperature at maximum speed.
2. Carefully remove the supernatant by pipetting without disturbing the pellet.
3. Add 1 ml 96–100% ethanol to the sample. Mix well by inverting the tube or by vortexing. Centrifuge at full speed for 2 min.
4. Carefully remove the supernatant by pipetting without disturbing the pellet.
5. Incubate the sample at room temperature (15–25°C) or at 37°C for 10 min until residual ethanol has evaporated.
6. Continue with Part II of the Protocol.

Heptane and methanol

1. Add 0.5 ml heptane to the sample. Vortex for 15 sec. Incubate for 10 min at room temperature (15–25°C).
2. Add 25 µl methanol. Vortex for 15 sec.
3. Centrifuge sample for 10 min at room temperature at 10 000 x g.
4. Carefully remove the supernatant by pipetting without disturbing the pellet.
5. Incubate the sample at room temperature (15–25°C) for 5 min to complete drying.
6. Continue with Part II of the Protocol.

Part II Isolation and purification of RNA

1. Suspend the dry pellet in 200 µl of buffer **Lyse ALL**. Mix thoroughly by vortexing

   - *Mechanical homogenization of samples suspended in Lyse ALL buffer increases the efficiency of RNA isolation. In case there is no access to a suitable mechanical homogenisator, it is still possible to continue with the protocol described below. A homogenisator is not a strictly required prerequisite for the further RNA isolation process.*

2. Add 15 µl of **Proteinase K**. Mix by pipetting or vortexing the tube.

   - *Depending on the type of embedded tissue, digestion with Proteinase K may not proceed to completion and traces of non-digested tissue may remain. Even partial digestion is sufficient to obtain satisfactory RNA yields.*

   - *For increasing the efficiency of digestion, mix the sample repeatedly by inverting the tube during incubation.*

3. Incubate the sample at 56°C for 20 min.

4. Continue incubating the sample at 80°C for 15 min.

   - *In case only one single heating block is available, leave the sample at room temperature during the temperature shift from 56°C to 80°C. Do not transfer the sample to the heating block, until the heating block has reached 80°C.*

   - *Heating the sample to 80°C allows partial reversion of nucleic acids modifications by formaldehyde and thus significantly increases the efficiency of purified RNA as template in RT-PCR applications.*

5. Snap cool the sample on wet ice for 2 min and centrifuge for 2 min at RT and maximum speed.

6. Carefully transfer 200 µl of the lysate to a new 1.5–2 ml Eppendorf-type tube (RNase-free) and add 300 µl of **RL** buffer. Mix thoroughly by pipetting. Following thorough mixing, add 600 µl of 96–100% ethanol and mix thoroughly again.

   - *In contrast to other EURx RNA extraction kits, it is not necessary to add β-mercaptoethanol to buffer RL in this kit. RNases are irreversibly denatured by formaldehyde during the fixation process. Thus, no further protecting additives are required.*

7. Carefully transfer 600 µl of the mixture to a **homogenization spin-column** placed in a 2 ml receiver tube. Centrifuge at maximum speed for 1 min. Remove the spin-column, pour off the supernatant and stick the spin column back onto the receiver tube.

   - *The homogenization column binds both nucleic acids, DNA and RNA. DNA is digested during a following DNase I digestion step in a liquid solution environment.*

8. Transfer the remaining mixture to the same **homogenization spin-column** placed in a 2 ml receiver tube. Centrifuge at maximum speed for 1 min.

9. Remove the spin-column, discard the flow-through and stick the spin column back onto the receiver tube.

10. Add 400 µl of **Wash RNA** buffer and centrifuge at maximum speed for 2 min.

    - *Be careful not to contaminate the sample while removing the spin-column from the receiver tube. Check, whether the bottom of the spin column is completely dry. If not, pour off any remaining supernatant and stick back spin-column onto the receiver tube. Spin down for one additional minute.*

11. Place spin-column into new receiver tube (1.5–2 ml) and add 80 µl **RNase-free water** directly onto the membrane.

12. Centrifuge at maximum speed for 1 min.

13. Remove the **homogenization spin-column**. Add 20 µl **DNRII** to the flow-through. Mix thoroughly by pipetting. Then, add 2 µl **DNase I**. Mix gently.

    - *DNase I is sensitive to physical denaturation. Be careful not to mix DNase I vigorously.*

14. Incubate the sample at room temperature (15–25°C) for 15 min.

    - *DNase I digestion in solution effectively eliminates remaining DNA.*

15. Following **DNase I** digestion, add 200 µl **RL** buffer to the sample. Mix well and add 360 µl 96–100% ethanol. Mix thoroughly again.

16. Apply the sample, including any precipitate, to the **RNA binding spin-column** placed in a 2 ml receiver tube. Centrifuge for 1 min at 11 000 x g.

17. Remove spin-column, discard flow-through and stick spin column back onto receiver tube.

18. Add 600 µl of **Wash RNA** buffer and centrifuge at 11 000 x g for 1 min.

19. Remove spin-column, discard flow-through and stick spin column back onto receiver tube.

20. Add 300 µl of **Wash RNA** buffer and centrifuge at 11 000 x g for 2 min.

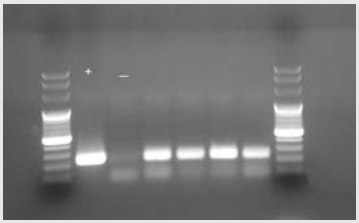
    - *Be careful not to contaminate the sample while removing the spin-column from the receiver tube. Check, whether the membrane of the spin column is completely dry. If not, pour off any remaining supernatant and stick back spin-column onto the receiver tube. Spin down for one additional minute.*

21. Place spin-column into new receiver tube (1.5–2 ml) and add 30 µl **RNase-free water** directly onto the membrane.

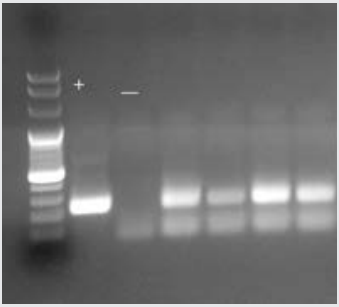
22. Centrifuge for 1 min at 11 000 x g.

23. Remove spin-column, cap the receiver tube. RNA is ready for analysis/manipulations. Isolated RNA can be stored either at 2–8°C (preferred) or at -20°C.

Examples of RT-PCR reactions performed on the basis of RNA isolated from paraffin-embedded tissues (FFPE).



Gen GAPDH RT-PCR results, 240 bp product. RT-PCR was performed using EURx OneStep RT-PCR kit (EURx cat. no. E0803) and 4 µl (100 ng) of RNA isolated as described in protocol (xylene option). Four different paraffin embedded bone samples with myeloma tumor. Perfect™ 100 bp DNA Ladder (EURx cat. no. E3134). (+) RNA from human leukocytes as a control.



Gen GAPDH RT-PCR results, 240 bp product. RT-PCR was performed using EURx OneStep RT-PCR kit (EURx cat. no. E0803) and 4.5 µl (120 ng) of RNA isolated as described in protocol (xylene option). Four different paraffin embedded breast samples. Perfect™ 100 bp DNA Ladder (EURx cat. no. E3134). (+) RNA from human leukocytes as a control.

Safety Information

Lyse ALL

Warning

H319 Causes serious eye irritation.
P280 Wear protective gloves/protective clothing/eye protection/face protection.
P305+**P351**+**P338** If in eyes: rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337+**P313** If eye irritation persists: Get medical advice/ attention.

RL

Warning

H302+**H332** Harmful if swallowed or if inhaled.
H412 Harmful to aquatic life with long lasting effects.
P273 Avoid release to the environment.
P301+**P312** If swallowed: call a poison center/ doctor/... if you feel unwell.
P304+**P340** If inhaled: remove person to fresh air and keep comfortable for breathing.
EUH032 Contact with acids liberates very toxic gas.

Proteinase K

Danger

H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.
P261 Avoid breathing vapours/spray.
P304+**P340** If inhaled: remove person to fresh air and keep comfortable for breathing.
P342+**P311** If experiencing respiratory symptoms: call a poison center or doctor/ physician.

Wash RNA

Danger

H225 Highly flammable liquid and vapour.
H319 Causes serious eye irritation.
P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
P280 Wear protective gloves/protective clothing/eye protection/face protection.
P305+**P351**+**P338** If in eyes: rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P403+**P235** Store in a well-ventilated place. Keep cool.
P337+**P313** If eye irritation persists: Get medical advice/ attention.

GeneMATRIX is synthetic, new generation DNA- and RNA-binding membrane, selectively binding nucleic acids to composite silica structures.

Novel binding and washing buffers are developed to take full advantage of GeneMATRIX capacity, yielding biologically active, high-quality nucleic acids. Matrix is conveniently pre-packed in ready-to-use spin-format. Unique chemical composition of the matrixes along with optimized construction of spin-columns improve the quality of final DNA or RNA preparation. To speed up and simplify isolation procedure, the key buffers are colour coded, which allows monitoring of complete solution mixing and makes purification procedure more reproducible.

As a result, we offer kits, containing matrixes and buffers that guarantee rapid, convenient, safe and efficient isolation of ultrapure nucleic acids. Such DNA or RNA can be directly used in subsequent molecular biology applications, such as: restriction digestion, dephosphorylation, kinasing, ligation, protein-DNA interaction studies, sequencing, blotting, in vitro translation, cDNA synthesis, hybrydization among others. Additional advantage is reproducibility of matrix performance, as component preparation is carried at Eurx Ltd.

GeneMATRIX FFPE RNA Purification Kit is designed for rapid purification of total RNA enriched with small RNA molecules, shorter than 200 nucleotides from formalin-fixed, paraffin-embedded tissue sections.

Firstly, all paraffin is removed from FFPE tissue sections by using one of deparaffinization method. Next, samples are incubated in an optimized lysis buffer, which contains Proteinase K, to release RNA from the sections. A short incubation at a higher temperature partially reverses formalin crosslinking of the released nucleic acids, improving RNA yield and quality as well as RNA performance in downstream enzymatic assays. The lysate is then transferred to specially designed mini spin-columns. These mini spin columns bind nucleic acids, remove impurities and prepare the samples for digestion with DNase I, which in the next stage effectively eliminates DNA. Addition of ethanol creates the proper conditions for selective binding of RNA to the membrane GeneMATRIX. Following adjustment of binding conditions, the samples are applied to RNA binding spin-columns. All RNA molecules are adsorbed to the column matrix and contaminants are efficiently washed away. High-quality RNA is then eluted in RNase-free water. Special columns containing membranes with improved binding performance allows to reduce the elution volume and finally increase concentration of RNA. The maximum binding capacity of columns is up to 100 µg total RNA. Isolated RNA is ready for downstream applications without the need for ethanol precipitation.

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