Isolation

miRCURY™ RNA Isolation Kit FFPE

Instruction manual v1.0

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EXIQON

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Table of contents

Product summary . . . 3
miRCURY™ RNA Isolation Kit – FFPE samples . . . 3
Additional required material . . . 3
Product description . . . 4
Storage and product stability . . . 7

Protocol overview . . . 8
Before starting the experiment . . . 8
Protocol & Notes . . . 9

Tips and troubleshooting . . . 12

Appendix A . . . 16

Related products . . . 17

Product summary

miRCURY™ RNA Isolation Kit – FFPE samples

The miRCURY™ RNA Isolation Kit – FFPE samples consists of the components listed in Table 1.

Table 1.

Kit Components (50 isolations)	Amount supplied
Paraffin Dissolver	60 mL
Lysis Buffer B	10 mL
Precipitation Buffer C	1 mL
Binding Buffer D	60 mL
DNase Buffer E	7 mL
Wash buffer F (concentrate)*	25 mL
H2O (RNase free)	13 mL
rDNase, RNase free (lyophilized)*	1 vial
Proteinase K	0.8 mL
RNA Mini Spin Columns FFPE	50
Collection tubes (1.5 mL)	1x50
Collection tubes (2.0 mL)	1x50

*Preparation of working solution required. See Protocol & Notes prior to use.

Additional required material

- 100% Ethanol
- Benchtop microcentrifuge
- Pipettes (+ RNase free tips)
- Vortexer / multi-vial vortex shaker
- Refrigerator /ice bath
- Heating blocks 56°C and 80°C

Product description

Exiqon's miRCURY™ RNA Isolation Kit – FFPE, provides a rapid method for the isolation and purification of RNA from formalin-fixed paraffin-embedded (FFPE) tissue samples which are commonly used for histopathological analysis. The sample blocks of interest may be either fresh or archived.

The miRCURY™ RNA Isolation Kit – FFPE contains the buffers, enzymes and column matrix needed for extraction of RT-PCR grade RNA for 50 samples.

The purification is based on 6 main steps - starting with a non-toxic deparaffinization step followed by a Proteinase K digest of the tissue. After adjustment of the binding conditions the lysate is applied to the silica matrix of the extraction column. Potential residual DNA is digested directly on-column and the RNA is purified by two wash steps prior to the elution.

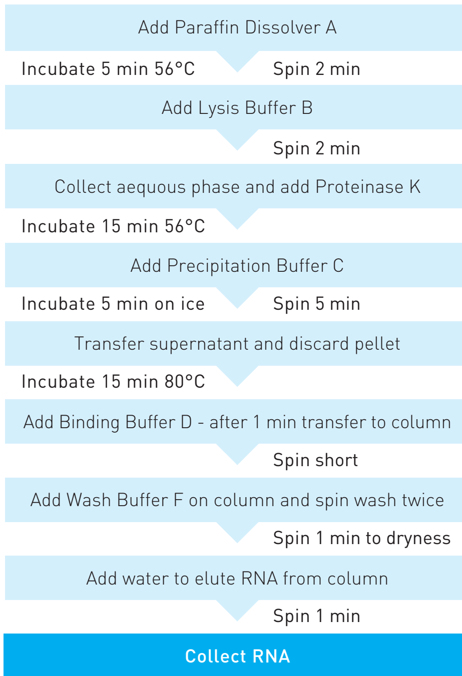
The extraction is performed at room temperature.

The resulting RNA sample should be stored at -20°C, ideally long-term at -80°C.

The protocols consist of 6 characteristic steps (see also Figure 1):

1. Non-toxic deparaffinization
2. Proteinase K digest
3. Adjusting binding conditions
4. On column DNase digest
5. Washing
6. Elution

Figure 1. Protocol overview of the miRCURY™ RNA Isolation Kit – FFPE.



Important note - cautions

Ensure that a suitable lab coat, disposable gloves and protective goggles are worn and standard safety precautions are followed when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs).

Exiqon's miRCURY™ RNA Isolation Kit – FFPE is recommended to isolate total RNA from thin (3-20 µm) sections of formalin-fixed paraffin-embedded (FFPE) tissue or of formalin-fixed tissue where the deparaffinization step of the protocol consequently is simply omitted. We recommend using no more than 5 mg of tissue per extraction which roughly corresponds to 5 x 10 µm sections from a 1 cm^2 tissue block. While we recommend 10 µm sections for the ease of handling the actual thickness can be varied between 3 to 20 µm if necessary.

RNA yield is not only dependent on sample type and tissue but also on tissue handling prior to fixation, the fixation procedure itself and the embedding and storage time and conditions. A highly standardized procedure for acquisition of specimen, FFPE sample preparation and maintenance of the samples collection is therefore key for any authoritative downstream experiment using the RNA obtained from the miRCURY™ RNA Isolation Kit – FFPE. This is especially true as the degree of residual crosslinking will not be detected by normal RNA QC procedures.

The RNA will show fragmentation with a size distribution from 15 to 5000 nt. As a sign of aging of the sample block RNA often shows further degradation to fragments of 100 to 300 nt. size. However best sample acquisition regiment, fixation and embedding procedure can result in RNA length larger than 5000 nt.

The nicking and partial degradation of the RNA obtained from FFPE samples prevents the use of standard QC procedures. As one example RIN values as quality cutoff criteria lose their predictive value for FFPE derived RNA. However an FFPE RNA quality factor based on the fragment size distribution (smear analysis) can be used to characterize the RNA quality of FFPE RNA after a bioanalyzer run (see Tips and Troubleshooting). Please also note that microRNA has been shown to give robust and biological significant signatures even from RNA showing severe FFPE induced degradation.

Table 2.

Kit Specifications	
Column matrix	Silica membrane
Maximum starting material supported	1-10 sections with up to 5mg tissue total
Yield	Variable
Elution volume	5-30 µL
Time to complete 6 purifications	70-90 minutes

Storage and product stability

Lyophilized DNase and Proteinase K should be stored at 4°C upon arrival (stable for >6 months).

All other solutions should be kept at room temperature (stable for >6 months). Storage at lower temperature can cause salt precipitation in the buffers. If this occurs incubate the buffer at 30-40°C and mix well to resuspend.

Protocol overview

Before starting the experiment

Working with RNA

RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. If working with RNA it is recommended as a first step to create an RNase-free environment following the precautions below.

- The RNA area should be located away from microbiological work stations.
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination.
- There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only.
- All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water.
- Clean all surfaces with commercially available RNase decontamination solutions.
- When working with purified RNA samples, ensure that they remain on ice.

Centrifugation procedures

All centrifugation steps in this protocol are carried out in a benchtop microcentrifuge at room temperature. Various speeds required for different protocol steps, are indicated in g-force. If your centrifuge has no possibility to adjust for g-force (RCF) the correct RPM can be calculated using the formula:

$$\text{RPM} = \sqrt{\frac{\text{RCF}}{(1.118 \times 10^{-5})\,(r)}}$$

where RCF = required gravitational acceleration (relative centrifugal force in units of g); r = radius of the rotor in cm; and RPM = the number of revolutions per minute required to achieve the necessary g-force.

Protocol & Notes

General recommendations when working with FFPE samples

Notes Prior to Use:

- Make sure that the tissue sample collection and treatment and storage up to this point has been as uniform as possible amongst the individual samples
- Any preparation is highly dependent on the amount of starting material. The standard protocol is flexible to extract RNA from starting material up to 5 mg

Important note - Prepare working solutions before proceeding to manual

rDNase: Prepare a working solution of rDNAse by adding 3 mL DNase Buffer E to the rDNase vial. Incubate at room temperature for 1 min. Swirl gently to redissolve completely but avoid vigorous mixing to minimize mechanical shearing which could affect enzyme quality. Aliquot and store at -20°C for later use. Avoid repeated freeze thawing. The working solution is stable for at least 6 months.

Wash Buffer F: add 100 mL 96-100% Ethanol to the concentrate. Mark bottle after addition of Ethanol. Store at room temperature.

Section 1. RNA isolation from less than 5 mg tissue FFPE or Formalin Fixed sample

Notes Prior to Use:

Before getting started please ensure that the DNase and Wash Buffer F were prepared accordingly. Set incubators to 56°C (for deparaffinization/lysis) and 80°C (for decrosslinking). If working with formalin fixed samples (without paraffin), skip step 2 and 4 and the centrifugation in step 3.

Step	Procedure
Step 1 Sample preparation	Collect FFPE sections in a 1.5 mL microcentrifuge tube (not provided with the kit)
Step 2 Deparaffinization	Add 1 mL of Paraffin Dissolver A to the sample and incubate 5 minutes at 56°C to melt the paraffin. Vortex the hot sample. ***Note:*** *make sure the paraffin is dissolved completely and mixed well with the buffer.* Spin 2 minutes at 16,000 x g
Step 3 Lysis	*Check Lysis Buffer B for precipitation. If necessary heat to 30-40°C to resuspend any precipitate and mix thoroughly.* Add 140 µl Lysis Buffer B. Keep the phases separated. DO NOT MIX! Spin 2 minutes at 16,000 x g
Step 4 Remove supernatant	Remove the supernatant with a pipet. ***Note:*** *Slight residues of Paraffin Dissolver A do not affect the following steps.*
Step 5 Proteinase K treatment	Add 12 µl Proteinase K. Mix by flicking or gentle pipetting. DO NOT VORTEX! Incubate 15 minutes at 56°C. *If tissue structure is then still visible prolong the incubation until the sample is digested.*
Step 6 Precipitation	Add 12 µL Precipitation Buffer C and vortex briefly. Incubate 5 minutes on ice / cool block. Spin 5 minutes at 16,000 x g.

Step 7 Incubation	Transfer supernatant to a new microcentrifuge vial (1.5 mL, not provided). Incubate exactly 15 minutes at 80°C. *Shorter time or lower temperature will lead to incomplete de-crosslinking. Longer time or higher temperature may affect RNA quality.*
Step 8 Adjust binding conditions	Add 400 μL Binding Buffer D, vortex briefly 3 seconds and incubate 1 minute at room temperature.
Step 9 Bind RNA to column	Place a spin column in a 2.0 mL collection tube and load the sample on the column. Spin 30 seconds at 16,000 x g. Discard flow through. Reuse collection tube with the spin column.
Step 10 Wash	Add 400 μL Wash Buffer F to the spin column. Spin 30 seconds at 16,000 x g. Discard flow through. Reuse collection tube with the spin column.
Step 11 Wash	Add 200 μL Wash Buffer F to the spin column. Spin 1 minute at 16,000 x g to dry the membrane completely. In case of touching of the flow through and nozzle of the column discard flow through and centrifuge again.*
Step 12 Elution	Place the column in a new 1.5 mL collection tube add 20 μL (For higher concentration use down to 5μL or up to 30 μL for higher yield) RNase free H2O to the column. Incubate for 1 minute at room temperature. Centrifuge 1 minute at 16,000 x g.

*see appendix A for the optional on column DNase step following step 11

Tips and troubleshooting

Poor RNA quality or yield

Poor specimen quality

Sample quality and uniformity in handling is key for any reliable downstream results. Further reading and recommendations regarding this topic:

- Castiglione F. et al.: Appl Immunohistochem Mol Morphol. 2007 Sep;15(3):338-42
- Chung J.Y et al.: J Histochem Cytochem. 2008 Nov;56(11):1033-42. doi: 10.1369/jhc.2008.951863. Epub 2008 Aug 18.
- Leyland-Jones et al: J Clin Oncol. 2008 Dec 1;26(34):5638-44. doi: 10.1200/JCO.2007.15.1712. Epub 2008 Oct 27.
- Von Ahlfsen et al.: PLoS One. 2007 Dec 5;2(12):e1261.
- Van Maldegem et al: Diagn Mol Pathol. 2008 Mar;17(1):51-8. doi: 10.1097/PDM.0b013e31814b8866.

RNA quality assessment

In order to characterize the sample quality of FFPE samples Illumina developed a value which characterizes the RNA quality based on the percentage of RNA with a nucleotide size larger than 200 nt (DV_{200}).As an example it is recommended to use samples with a DV_{200} > 33 % for NGS library preparations. In RNA sequencing experiments the D_{V200} value has shown to be more reliable for determining RNA quality of FFPE samples as compared to the otherwise widely used RNA Integrity Number (RIN) approach. For miRNA profiling such FFPE RNA sample quality analysis turned out to be of minor importance.

http://res.illumina.com/documents/products/technotes/technote-truseq-rna-access.pdf

http://res.illumina.com/documents/products/technotes/technote-expression-analysis-ffpe-samples.pdf

RNase contamination

Create an RNase free environment. Wear gloves throughout the whole RNA isolation process and change gloves frequently. Use sterile tubes and filter strips. Glassware should be oven baked >2hrs at 250°C.

Reagents not handled or stored properly

Dispense the volumes indicated in the manual and follow the instructions given. Remember to prepare aliquots and working solutions and store kit components as indicated.

Insufficient Proteinase K digestion

Depending on the sample material and initial fixation procedures the optimal digestion time can vary from 15 minutes to 3 hours. The incubation time should be prolonged when tissue structure is still visible. Even though not generally recommended, the samples can be digested overnight if the majority of the tissue did not get dissolved during an initial 3 hour incubation. In general we recommend the following alternative protocol for difficult tissues, replacing step 5-7 in the general protocol:

Step 5b	Add 12 µl Proteinase K. Mix by flicking or gentle pipetting. DO NOT VORTEX! Incubate 90 minutes at 56°C. *If tissue structure is then still visible prolong the incubation until the sample is digested.*
Step 6b	Add 12 µL Precipitation Buffer C and vortex briefly. Incubate 5 minutes on ice. Spin 5 minutes at 16,000 x g.
Step 7b	Transfer supernatant to a new micorcentrifuge vial (1.5 mL, not provided).

Working with a vacuum manifold

Depending on the vacuum manifold residual Paraffin Dissolver A can corrode or at least turn certain plastic opaque. To rule out any contamination, a slightly modified protocol replacing steps 3, 4 and 5 is suggested:

Step 3 Wash	Remove the supernatant with a pipet. Add 1 ml of 100 % ethanol to the pellet and mix by vortexing. Spin 2 minutes at 16,000 x g
Step 4 Dry	Remove the supernatant with a pipet. Spin 30 sec at 16,000 x g and remove any residual liquid with a pipet and let the pellet dry completely (10 - 20 minutes).
Step 5 Lysis / Proteinase K treatment	*Check Lysis Buffer B for precipitation. If necessary heat to 30-40°C to resuspend any precipitate and mix thoroughly.* Add 140 µl Lysis Buffer B. Then add 12 µl Proteinase K. Mix by flicking or gentle pipetting. DO NOT VORTEX! Incubate 15 minutes at 56°C. If tissue structure is then visible prolong the incubation until the sample is digested.

The vacuum manifold can then be used in steps 9 to 11 placing the columns on the vacuum manifold for loading and washing.

Contamination with DNA

Improper DNA removal

Reconstitute and store the DNase as indicated. Do not exceed the maximal amount of FFPE starting material. When performing the on-column digestion, apply the solution onto the center of the silica membrane and close the lid to press the liquid into the matrix. Additionally a secondary DNase step can be performed.

Secondary DNase step

If it is necessary to reduce the residual DNA contamination even further proceed as follows: Add 1/10 volume of DNAse to the eluted RNA (step 12), incubate 10 minutes at 37°C then incubate 5 minutes at 75 °C. Add a further cleanup step to remove DNase when needed.

Sub-optimal performance of RNA in downstream experiments

Salt or ethanol carry-over

Make sure the nozzle of the spin column does not touch the flow through fraction after the second wash step. Follow centrifugation speed and times indicated to remove the wash buffer completely. Make sure the wash buffer is at room temperature to enable efficient salt removal. If your subsequent RT-qPCR shows signs of inhibition, titrate the RNA to find a suitable lower input amount.

RNases

For short periods of time RNA can be stored at -20°C or below. To minimize the risk of RNA degradation long term storage should occur at -80°C if possible

Appendix A

On-column DNA removal

Step 11a DNase treatment	Add 25 µL rDNase on the column matrix and incubate at room temperature for 15 minutes.
Step 11b Binding	Add 50 µL Binding Buffer D and incubate at room temperature for 1 minute. Spin 30 seconds at 16,000 x g. Discard flow through. Reuse collection tube with the spin column.
Step 11c Wash	Add 400 µL Wash Buffer F to the spin column. Spin 30 seconds at 16,000 x g. Discard flow through. Reuse collection tube with the spin column.
Step 11d Wash	Add 200 µL Wash Buffer F to the spin column. Spin 1 minute at 16,000 x g. Discard flow through and centrifuge again for 5 minutes at 16,000 x g to dry the membrane. Continue with step 12...

Related products

Exiqon offers specialized RNA isolation kits enabling preparation from larger amounts of human/animal tissues as well as cells and plants and downstream application products to analyze and verify the expression, function and spatial distribution of microRNAs:

miRCURY™ RNA Isolation Kit – Biofluids

Small RNA preparations from serum or plasma samples or from serum or plasma. Suitable for miRCURY LNA™ Universal RT microRNA PCR.

miRCURY™ RNA Isolation kit – Cell & Plant

Total RNA preparations from cultured animal cells, small tissue samples, blood, bacteria, yeast, fungi, bacteria and plants.

miRCURY™ RNA Isolation kit -Tissue

Get high quality total RNA suitable for miRCURY LNA™ Universal RT microRNA PCR or miRCURY™ LNA microRNA Array analysis in as little as 20 minutes. Total RNA preparations from 15-30 mg animal/human tissue.

miRCURY™ Exosome Isolation kit – Serum and Plasma and miRCURY™ Exosome Isolation kit – Cells, urine and CSF

High quality exosome isolation using a fast protocol for scalable isolation of exosmes from various biofluids. Exosome isolation using miRCURY™ Exosome Isolation Kits ensures seamless integration with the miRCURY™ RNA Isolation Kits and miRCURY LNA™ Universal RT microRNA PCR system.

miRCURY LNA™ Universal RT microRNA PCR

Exiqon's microRNA qPCR system offers the best available combination of performance and ease-of-use on the microRNA real-time PCR market. The combination of a Universal RT reaction and LNA™-enhanced PCR primers results in unmatched sensitivity and specificity. The Ready-to-use microRNA PCR panels enable fast and easy microRNA expression profiling. Take advantage of the tailored Universal RT microRNA PCR spike in kit to monitor the performance of your PCR.

miRCURY LNA™ microRNA Array, microarray kit

Pre-printed miRCURY LNA™ microRNA Array microarray slides, available in pack sizes of 3, 6 and 24 for hsa, mmu & rno and other species. The kit comes complete with hybridization and wash buffers as well as synthetic spike-in microRNAs.

miRCURY LNA™ microRNA Detection

For *in situ* hybridization and northern blotting of all annotated microRNAs. miRCURY LNA™ microRNA ISH Buffer Set (FFPE).

miRCURY LNA™ microRNA ISH Optimization kit (FFPE)

Complete kit with control probes and hybridization buffer for easy set up of microRNA *in situ* hybridization.

Literature citations

Please refer to miRCURY™ RNA Isolation Kit - FFPE when describing a procedure for publication using this product.

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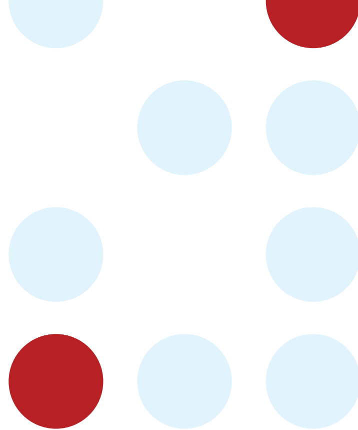

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