EchoLUTION™ FFPE RNA Kit

Spin column kits

USER MANUAL

REF

011-005-010

011-005-050

011-005-250

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1. INTENDED PURPOSE

The BioEcho EchoLUTION FFPE RNA Kit is intended for easy, fast, and efficient RNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue. The high yield and purity of total RNA obtained with the EchoLUTION FFPE RNA Kit allows use in several downstream applications, such as NGS, PCR, and qPCR without further processing.

The EchoLUTION FFPE RNA Kit is intended for research use only.

2. EXPLANATION OF THE KIT

The EchoLUTION FFPE RNA Kit is characterized by a tailored decrosslinking and lysis step followed by a single-step purification. Together, these steps reduce the overall extraction time drastically with consistent quality compared to state-of-the-art methods.

The key steps of the EchoLUTION FFPE RNA Kit procedure are:

1. FFPE decrosslinking
2. FFPE tissue lysis and removal of detergent and paraffin
3. Single-step RNA purification (see section 2.1)

In contrast to conventional methods, the benefits of the EchoLUTION FFPE RNA Kit are:

- Shorter processing time
- Fewer protocol steps
- Minor equipment and capital investment
- Less plastic waste
- Fewer toxic reagents (no usage of xylene or similar reagents during deparaffinization, no ethanol steps, in total less liquid waste)

For further kit specifications, see Table 1.

Table 1: ***Kit specifications***

Specification	Description
Sample type	FFPE tissue
Sample input	100–800 mm^2 area (thickness 10 µm) or 1–15 mg FFPE tissue
Sample condition	Formalin-fixed paraffin-embedded
Purified nucleic acid	RNA
Elution volume	~80 µL
Expected yields	Highly dependent on amount of starting material and tissue type

2.1. *Single-step purification principle*

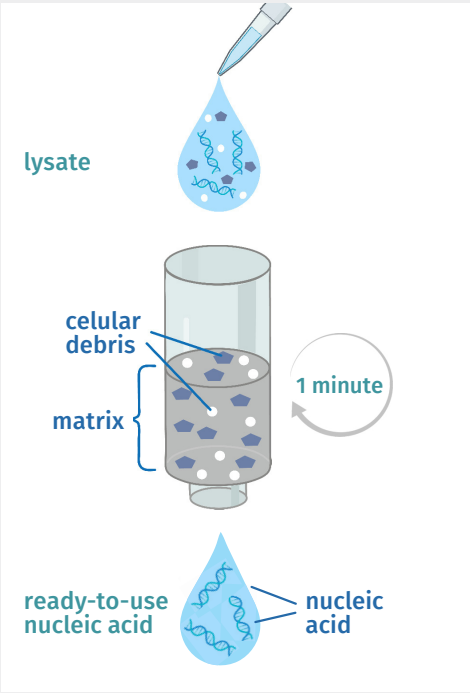
All BioEcho nucleic acid extraction kits are based on the EchoLUTION technology, which consists of tailored sample processing that includes lysis followed by single-step purification. The purification step works differently than conventional methods such as magnetic-bead and silica kits based on the bind–wash–elute method, and therefore needs only one centrifugation step.

The lysate is transferred onto the spin column or plate.

In a one-minute centrifugation step, nucleic acids pass through the purification matrix without interaction.

Impurities are held back and thereby completely removed.

The nucleic acids are in the flow-through and ready-to-use.



2.2. *General comments*

Comparison of EchoLUTION™ technology to silica methods—general aspects and handling

Using the EchoLUTION technology, nucleic acids are not bound to a membrane or magnetic beads and can migrate freely through the filter matrix. Unwanted components of the lysate are removed from the sample by remaining in the purification matrix.
The advantages of the EchoLUTION technology are:

1. No time-consuming washing steps
2. Easy handling
3. Reduced plastic waste

In contrast, silica technologies are based on the principle of concentration. Here, the nucleic acids present in the lysate bind to a silica surface (membrane, magnetic beads), while unwanted cell components are removed by repeated washing with chaotropic and alcohol-containing wash buffers. Eventually, the nucleic acids are eluted with an aqueous buffer in the desired volume. Due to the repeated washing steps, silica-based methods are time-consuming, labor-intensive, and environmentally unfriendly.

Handling of purification matrix

The EchoLUTION purification matrix within the Spin Columns must be kept undamaged to avoid short-circuit currents. Short-circuit currents result in the introduction of lysis components into the eluate and inadequate purification, which can lead to inhibition in downstream analysis. Therefore, when applying the lysate to the column **avoid touching the surface** of the filter matrix and **pipet the sample very slowly (ideally dropwise)**.
To guarantee proper handling of the Spin Columns, be sure to use the recommended *g*-force centrifuge settings. Most centrifuges offer the choice between rpm and *g*-force (rcf); if not, calculate the rpm corresponding to the required *g*-force using the calculator in the link or the QR code below:

www.geneinfinity.org/sp/sp_rotor.html

For support on suitable centrifuges, please contact us.

FFPE sample storage

In general, FFPE blocks can be stored at room temperature. For best RNA extraction results, samples should be stored at 2 to 8 °C or –20 to –70 °C.

Preparation of FFPE samples

Formalin fixation and paraffin embedding is a widely used and valuable process to preserve tissues. Many fixation protocols are available to match the wide range of applications in which FFPE tissue is used. Accordingly, there is no standard fixation protocol. However, the method used has a large influence on the quality of extracted nucleic acids and their performance in downstream applications. Several factors influence the quality of tissues and preservation. The

larger the biopsy, the more inhomogeneous the tissue preservation is likely to be. The amount of time after the biopsy is taken and before it is embedded in paraffin is also variable. Longer times before embedding can lead to degradation of proteins and nucleic acids contained in the tissue. In addition, the longer a biopsy is preserved in formalin, the greater is the degree of crosslinking. Crosslinking can be reversed with incubation at 90 °C.

Handling RNA

For high-quality RNA extraction, the samples must be free of ribonucleases (RNases), as these enzymes digest RNA very efficiently. In general, RNases are very active, stable, and difficult to inactivate. Even small amounts can digest sufficient RNA to drastically reduce the yield. To avoid RNase contamination of your samples, some things need to be considered. Always eliminate possible RNase contamination from any consumables you will use before starting your extractions. Make sure that the working area is free of RNases throughout the whole procedure to avoid introducing RNases into the samples.

Bench surfaces, laboratory equipment (e.g., pipettes and electrophoresis tanks) and non-disposable plasticware can be decontaminated using general laboratory reagents. Plasticware can be decontaminated with commercially available RNase-removing solutions.

In general, aseptic techniques should always be used when working with RNA to avoid contamination risk. The most common sources of RNases are dust and hands, as they can hold bacteria and molds. Therefore, always wear gloves while handling reagents and RNA samples. Replace gloves regularly and keep tubes closed when possible.

For long-term storage, purified RNA can be stored at –70 °C. Samples can be aliquoted to avoid repeated thaw and freeze cycles. When working with the RNA for downstream applications, thaw on ice and keep on ice while working.

RNA fragmentation

Formalin fixation and paraffin embedding crosslinks cellular components such as DNA, RNA, proteins, and enzymes to stop necrosis. The longer the FFPE sample is stored, the higher the degree of the resulting nucleic acid fragmentation. Furthermore, fragmentation happens at a higher rate at higher temperatures. Thus, on one hand, higher temperatures during the embedding process as well as during the decrosslinking step increase the degree of fragmentation. On the other hand, these higher temperatures can increase the nucleic acid yield. Therefore, you must balance the advantages and disadvantages according to your desired result. For optimal RNA extraction, the time of the decrosslinking incubation should take into consideration the sample age and length of formalin fixation, both of which also have an impact on fragmentation.

Excessive paraffin content

Large amounts of paraffin contained in a sample can make handling difficult and has a negative impact on yield and quality of RNA. For such samples, a reduction of the lysate volume and eluate volume is also to be expected. During sample preparation, as much surrounding paraffin as possible should be removed and only the “usable area” (shown in Figure 1, page 12) should

be used for RNA extraction. This is especially recommended for RNA extraction of small FFPE tissue biopsies contained in a large piece of paraffin.

3. MATERIALS

3.1. Materials provided

Table 2: ***Content of EchoLUTION FFPE RNA Kit***

Product number	011-005-010	011-005-050	011-005-250
Product name	EchoLUTION FFPE RNA Kit (10)	EchoLUTION FFPE RNA Kit (50)	EchoLUTION FFPE RNA Kit (250)
Reactions	10	50	250
Lysis Buffer FFPE (LB)	1 × 1.1 mL	1 × 5.5 mL	1 × 27.5 mL
TurboLyse Protease FFPE (P)	1 × 100 µL	1 × 500 µL	1 × 2.5 mL
Removal Solution FFPE (RS)	1 × 2 mL	1 × 10 mL	2 × 25 mL
DNase FFPE (D)	1 × 100 µL	1 × 100 µL	3 × 100 µL
Reaction Buffer DNase FFPE (RB)	1 × 1.5 mL	1 × 1.5 mL	3 × 1.5 mL
Low-TE Buffer (T)	1 × 1.2 mL	1 × 1.2 mL	2 × 1.2 mL
Spin Columns FFPE	10	50	250

3.2. Materials required but not provided

A. Microcentrifuge tubes

Always use nuclease-free tubes. Use a 2 mL tube for spin column preparation and a 1.5 mL tube for sample lysis and elution.

3.3. Optional materials

A. BioEcho Cap Puncher

Alternatively, the Cap Puncher can be used for convenient handling of Spin Columns. To purchase this item, use the product number 050-001-001.

B. Spin Column Adapter for Plate Centrifuges

If you want to use a plate centrifuge for Spin Columns and avoid the standing time of the Spin Columns mentioned in the protocol, we suggest using a swing-out rotor centrifuge with our Spin Column Adapter for Plate Centrifuges (product number 050-011-024).

3.4. Laboratory equipment needed

A. Microcentrifuge

Centrifugation can be performed in a microcentrifuge with a rotor for 2 mL reaction tubes. When using a plate centrifuge, please use our Spin Column Adapter for Plate Centrifuges (product number 050-011-024). The centrifuge needs to reach a speed of 1,000 x *g*. Note that for the phase separation at 19,000 x *g*, you need a microcentrifuge capable of reaching that speed.

B. Pipetting equipment

Pipetting can be performed using 10 µL and 200 µL single-channel pipettes.

C. Thermal shaker

The thermal shaker is used for the decrosslinking, tissue lysis, and removal of detergent and paraffin step. It needs to reach up to 90 °C and 1,400 rpm (e.g., Eppendorf® ThermoMixer® C). It is recommended to use two thermal shakers for the protocol.

D. Vortex mixer

A vortex mixer is required for lysate mixture.

4. STORAGE AND STABILITY

4.1. Kit and reagents

The EchoLUTION FFPE RNA Kit is shipped at ambient temperature except for the DNase FFPE, which is shipped cooled in a separate box.

Upon kit arrival, TurboLyse Protease FFPE should be stored at 2–8 °C and the DNase FFPE should be stored at –20 °C. The other kit components can be stored at 2–8 °C, but are also stable at room temperature (15–25 °C).

- If Removal Solution FFPE is stored at 2–8 °C, it may become solid. Please leave at room temperature till it becomes liquid again (30–90 minutes). If necessary, heat up to 56 °C to speed up the process.
- If Lysis Buffer FFPE is stored at 2–8 °C, a precipitate might form. Please leave the buffer at room temperature for a few minutes till the white precipitate is dissolved. If necessary, heat up to 56 °C for a few minutes to fully dissolve the precipitate.

DNase FFPE

The DNase FFPE needs to be frozen upon arrival and repeated thaw and freeze cycles should be avoided. If this cannot be avoided, consider to aliquot the DNase FFPE in nuclease-free tubes. Furthermore, while using the DNase FFPE, please keep the tube on ice.

4.2. *Storage and stability of purified nucleic acids*

For long-term storage of purified RNA, it is recommended storing the RNA samples at –70 °C. Avoid repeated thaw and freezing cycles, aliquot RNA if needed.

5. WARNINGS AND SAFETY INSTRUCTIONS

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. The Lysis Buffer FFPE (LB) requires nitril gloves (NBR). For more information, please refer to the instructions supplied with our safety data sheets (SDS). Please **contact us** for the SDS.

Table 3: ***EchoLUTION FFPE RNA Kit safety information***

Component	Hazardous component	GHS symbol	Hazard statements	Precautionary statements	Additional statement
Removal Solution FFPE	112-30-1 Decanol	 GHS07 **Warning**	H319, H412	P101; P102; P103; P264; P273; P280; P305+P351+P338; P337+P313; P501	—
TurboLyse Protease FFPE	Subtilisin	 GHS05 GHS07 GHS08 GHS09 **Danger**	H315; H318; H334; H335; H400; H411	P101; P102; P103; P261; P284; P305+P351+P338; P310; P405; P501	—

Hazard Statements

H315: Causes skin irritation.
H318: Causes serious eye damage.
H319: Causes serious eye irritation.
H334: May cause allergy or asthma symptoms or breathing difficulties if inhaled.
H335: May cause respiratory irritation.
H400: Very toxic to aquatic life.
H411: Toxic to aquatic life with long lasting effects.
H412: Harmful to aquatic life with long lasting effects.

Precautionary statements

P101: If medical advice is needed, have product container or label at hand.
P102: Keep out of reach of children.
P103: Read label and MSDS before use.
P261: Avoid breathing dust/fume/gas/mist/vapors/spray.
P264: Wash thoroughly after handling.

P273:	Avoid release to the environment.
P280:	Wear eye protection/face protection.
P284:	[In case of inadequate ventilation] wear respiratory 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.
P310:	Immediately call a POISON CENTER/doctor.
P337+P313:	If eye irritation persists: Get medical advice/attention.
P405:	Store locked up.
P501:	Dispose of contents/container in accordance with local/regional/national/international regulations.

6. DISPOSAL

Please follow local regulations regarding collection and disposal of hazardous waste and contact your waste disposal company to obtain information about laboratory waste disposal (waste code number 16 05 06). For more information, please refer to the instructions supplied with the safety data sheet (SDS). Please **contact us** for the SDS.
Dispose all biological samples and all liquid waste generated during the purification procedure as biohazardous waste.

A. Removal Solution FFPE and TurboLyse Protease FFPE

Very toxic or harmful to aquatic organisms with long-term effects. Discharge into the environment must be avoided. Do not allow undiluted or large quantities of the product to reach groundwater, watercourse or sewage system. Empty bottles may retain some product residues. Therefore, ensure the dispose of contents/container under local/regional/national/international regulations.

B. Other components and Spin Columns

No special measures for disposal are necessary. Components that have been in contact with potentially infectious material should be autoclaved. Used components may retain some buffer residues, which should be disposed of by local/regional/national/international regulations.
The storage buffer of the Spin Columns collected in the collection tube during the conditioning step can be disposed of in the sink or on a paper tissue.

7. PROTOCOL

This protocol has been developed to extract RNA from FFPE tissues using the EchoLUTION FFPE RNA Kit.

This is not a silica-based kit! Please read the instructions carefully before starting!

Preparation before starting:

- If Removal Solution FFPE (RS) and Lysis Buffer FFPE (LB) have been stored at 2–8 °C, please leave them at room temperature until the Removal Solution FFPE becomes liquid (up to 90 minutes) and no precipitate is visible in the Lysis Buffer FFPE (a few minutes). If necessary, heat up to 56 °C for a few minutes to speed up the process.

- Vortex EchoLUTION Spin Columns thoroughly upside-down to homogenize the matrix and remove air bubbles. If necessary, flick or gently spin down by hand until it is free of air bubbles. Place each tube in a 2 mL reaction tube (not provided) and let them stand to sediment the matrix until step 4.
  IMPORTANT NOTE: *For improved sedimentation of the matrix, we recommend that this step is performed upon receipt of the kit. Store them in upright position. The columns should stand for at least 60 minutes.*
- Heat the thermomixer to 90 °C; if available, heat a second thermomixer to 60 °C.
- Set the microcentrifuge to 1,000 x *g*.
  IMPORTANT NOTE: *Choose the x g (rcf) setting, not rpm.*
- Prepare nuclease-free 1.5 mL microcentrifuge tubes (not provided, preferably safe lock) for each sample.

1. FFPE tissue transfer

- When using FFPE blocks, cut sections using a microtome. Use of FFPE sections on slides is also possible. For both FFPE blocks and FFPE sections avoid transferring surrounding paraffin (Figure 1). For each sample, use 1–15 mg FFPE tissue or a minimum of 100 mm^2 and not more than 800 mm^2 of sections up to 10 µm thickness. Transfer the FFPE tissue into a 1.5 mL microcentrifuge tube.
  NOTE: *An excessive amount of surrounding paraffin can negatively influence the purification process resulting in reduced yield. Therefore, remove as much surrounding paraffin as possible. RNA yield is influenced by the FFPE tissue type and storage time. To ensure adequate yield from different FFPE tissues, adjust the amount of starting material accordingly.*
  NOTE: *Sections thicker than 10 µm can result in lower yields due to inefficient removal of paraffin.*

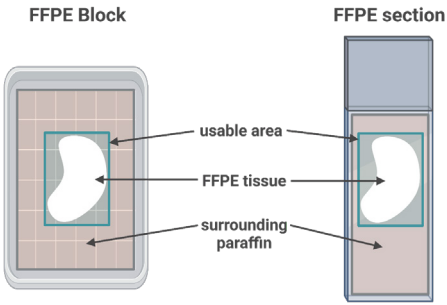


Figure 1: ***Schematic representation of an FFPE block and an FFPE section. Light brown: areas with paraffin only; white: area with fixed tissue; light green: area to be used for FFPE tissue extraction.***

2. *FFPE decrosslinking*

- Add 110 µL Lysis Buffer FFPE to each sample.
- Incubate samples at 90 °C for 40 minutes in a thermal shaker with constant shaking at 1,400 rpm and ensure that the tissue pieces are at the bottom.
  NOTE: *If you are concerned about highly fragmented RNA, you can reduce the incubation time to 30 minutes, but not below. Decreasing the incubation time might lead to less fragmentation but also less yield.*
- Briefly spin the tubes to remove any droplets from the lid.
- Remove tubes from thermal shaker and let them cool down to room temperature (approximately 5 minutes).
  IMPORTANT NOTE: *Do not cool the tubes on ice.*
  NOTE: *If a second thermal shaker is not available, set the thermal shaker to 60 °C for step 3.*
  OPTIONAL: *Briefly centrifuge at low speed (e.g., 10 seconds at 1,000 x g) to collect the drops from the lid.*

3. *FFPE tissue lysis, and removal of detergent and paraffin*

- Add 10 µL TurboLyse Protease FFPE (P) directly into the sample.
- Add 200 µL of Removal Solution FFPE to the sample and briefly vortex.
  IMPORTANT NOTE: *No master mix possible, because the two reagents do not mix.*
- Incubate at 60 °C for 30 minutes in a thermal shaker with constant shaking at 1,400 rpm for tissue lysis. Proceed with step 4 during incubation.
- Incubate at 90 °C for 5 minutes in thermal shaker with constant shaking at 1,400 rpm for protease inactivation.
  NOTE: *Extending the incubation time at 90 °C to 10 minutes can improve results depending on the tissue and age of the sample. However, extending the incubation time can also lead to more fragmentation*

of RNA.

- After incubation, set thermal shaker to 37 °C for DNase digestion.

4. *Spin Column preparation & conditioning*

- Loosen the cap of the Spin Column by ½ a turn and snap off the bottom.
- Place Spin Column back into the 2 mL reaction tube.

- Centrifuge 1 minute at 1,000 x *g*, and discard flow-through.
- Place the Spin Column in a fresh 1.5 mL reaction tube (not provided).

ALTERNATIVELY: *You can use the BioEcho Cap Puncher to open the column (not supplied, product number 050-001-001). To use the Cap Puncher correctly, punch a hole into the column cap and lift the column together with the Cap Puncher out of the 2 mL reaction tube. Snap off the bottom closure of the column and detach the Cap Puncher. Place the punched Spin Column back into the 2 mL reaction tube. Centrifuge 1 minute at 1,000 x g, discard flow-through and place the Spin Column in a fresh 1.5 mL reaction tube.*

5. *RNA purification*

- After incubation, cool down the samples (from step 3) for 3 minutes at room temperature.
- Centrifuge the samples for 4 minutes at maximum speed (minimum 19,000 x *g*) to separate the three phases (lower phase: aqueous, interphase: paraffin, upper phase: hydrophobic).

DNase digestion:

- Transfer the lower aqueous phase (~ 80 µL, lysate containing the RNA) into a new 1.5 mL microcentrifuge tube.

  NOTE: *Small amounts of the interphase containing paraffin or the hydrophobic upper phase have no negative effect on DNase digestion.*

- *Prepare a* DNase *master mix for the number of samples with 10 % excess (according to Table 4), mix gently and keep cold.*

Table 4: **DNase FFPE *master mix***

Number of samples	1	10 (+10%)	Your calculations
DNase FFPE (D)	1 µL	11 µL	
Reaction Buffer DNase FFPE (RB)	10 µL	110 µL	
Final volume	**11 µL**	**121 µL**	

- Add 11 µL of DNase FFPE master mix to the lysate and homogenize by inverting

several times. Briefly spin. Incubate the sample 15 minutes at 37 °C and 300 rpm. Then incubate another 10 minutes at 65 °C and 300 rpm to inactivate the DNase.

NOTE: *If you are concerned about further fragmentation, the heat-inactivation of the DNase can be skipped, but keep in mind that some DNase might still be present in the eluate and might interfere with your downstream application. For example in RT-qPCR, where $MgCl_2$ is involved, the DNase might become active and digest the cDNA. Therefore we recommend the heat inactivation.*

- Transfer the sample onto the prepared Spin Column.

  IMPORTANT NOTE: *Pipet slowly, drop-by-drop, and vertically onto the middle of the Spin Columns to not destroy the matrix surface. Do not touch the matrix bed with the pipette tip during sample loading!*

  NOTE: *If you have used the Cap Puncher make sure that you pipette vertically through the hole in the lid. Do not punch the pipette tip into the matrix while loading the lysate onto the EchoLUTION Spin Column.*

- If you did not use the Cap Puncher, close the cap of the Spin Column and loosen the cap again by ½ a turn.

- Centrifuge the loaded column for 1 minute at 1,000 x *g*.
- Purified RNA is in the flow-through.

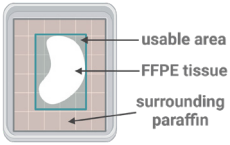
IMPORTANT NOTE: The extracted RNA can be stored or used directly. For long-term storage, place your RNA samples at –70 °C. For spectrophotometric analysis, use the Low-TE Buffer (T) supplied with the kit as blank.

7.1. Quick protocol EchoLUTION™ FFPE RNA Kit

IMPORTANT NOTE: *Please use the quick protocol only after you have read and understood the complete user manual. Read the* ***preparation before starting*** *section of chapter 7 (Protocol).*

1. FFPE tissue transfer

- Transfer 100–800 mm² (thickness 10 µm) or 1–15 mg FFPE tissue from usable area into a 1.5 mL tube.



2. FFPE decrosslinking

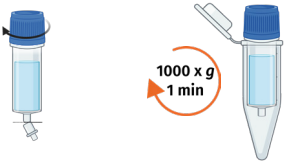
- Add 110 µL LB.
- Incubate at 90 °C for 40 min at 1,400 rpm.
- Briefly spin to remove droplets from the lid.
- Cool down to RT (~5 min).

3. FFPE tissue lysis, detergent and paraffin removal

- Add 10 µL P directly into the sample.
- Add 200 µL RS and briefly vortex.
- Incubate at 60 °C for 30 min at 1,400 rpm.
- Incubate at 90 °C for 5 min at 1,400 rpm.

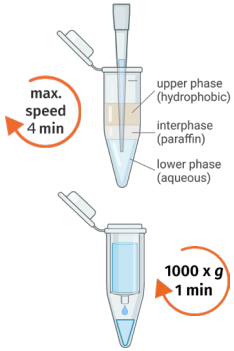
4. Spin Column preparation & conditioning

- Use the prepared sedimented Spin Columns (see *preparation before starting*).
- Loosen the cap of the Spin Column ½ a turn and snap off the bottom.
- Place Spin Column in a 2 mL reaction tube.
- Centrifuge 1 min at 1,000 x *g*.
- Discard flow-through.
- Place Spin Column in a new 1.5 mL reaction tube



5. RNA purification

- Cool down samples from step 3 for 3 min at RT.
- Centrifuge 4 min at maximum speed (minimum 19,000 x *g*).
- Transfer lower aqueous phase to a new 1.5 mL tube.
- Add 1 µL D and 10 µL RB and homogenize by inverting. Briefly spin.
- Incubate 15 min at 37 °C and 300 rpm followed by 10 min at 65 °C and 300 rpm
- Transfer the sample onto prepared Spin Column. Pipet slowly, drop-by-drop onto the middle of the column without touching the matrix.
- Centrifuge 1 min at 1,000 x *g*.
- Purified RNA is in the flow-through and ready-to-use.



8. QUALITY CONTROL

Following the BioEcho Quality Management System, each lot of EchoLUTION FFPE RNA Kit is tested against predetermined specifications to ensure consistent product quality. To request the Certificate of Analysis (CoA), please contact us.

9. TROUBLESHOOTING

Observation	Comments and suggestions
RNA yield and concentration is low or **Poor performance in downstream experiments**	**Low RNA content** Dependent on the tissue type, some samples could lead to a low RNA yield (e.g., bladder, bone). To ensure a succesful workflow, you could use a reference sample or another extraction method to evaluate the results in comparison. **Tissue with small surface area** To achieve a higher nucleic acid concentration, the amount of Lysis Buffer FFPE can be reduced from 110 µL to a minimum of 70 µL. This is beneficial for samples with a small total surface area. However, the reduction may result in more difficult handling and a lower yield, especially if the FFPE sample contains a lot of paraffin. If the volume of the Lysis Buffer FFPE is reduced, please ensure that at least 50 µL is applied to the Spin Column in step 5. Please note that the volume reduction does not necessarily lead to a proportional increase of the nucleic acid concentration in relation to the reduced volume. **Delayed embedding** It is crucial to preserve the tissue as fast as possible. A delay can lead to degradation of the tissue resulting in lower RNA yields. **Insufficient preservation** Fixation time is a critical factor for RNA yield and quality and highly depends on tissue size and fixation method. The larger the biopsy, the more inhomogeneous the tissue preservation can be and might thereby result in lower yields.

RNA yield and concentration is low

or

Poor performance in downstream experiments

(continuation)

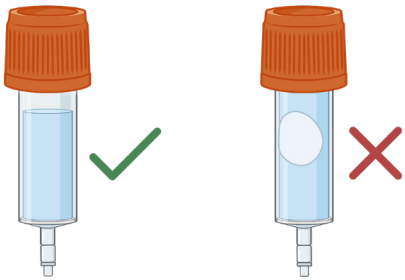
Sample storage
Sample storage time can affect RNA yield and integrity. For long-term storage, FFPE samples should be kept at 2 to 8 °C or –20 to –70 °C. FFPE sections on slides are exposed to air, which has a negative influence on the RNA.

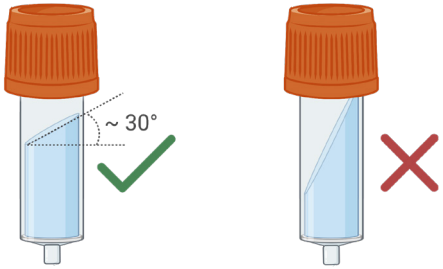
Sample input
Always use a sufficient amount of sample to ensure appropriate experimental conditions. Use 1–15 mg FFPE tissue or a minimum of 100 mm^2 and not more than 800 mm^2 of sections up to 10 µm thickness.

Centrifuge settings
Most centrifuges offer the choice between rpm and *g*-force (rcf). To calculate the rpm, see chapter 2.2. Always make sure to stick to the correct time mentioned in the protocol to avoid insufficient elution of RNA.

Large amounts of paraffin
An excessive amount of surrounding paraffin can negatively influence the purification process resulting in decreased yield and eluate volume. Therefore, remove as much surrounding paraffin as possible and use only the "usable area" (shown in Figure 1). If your sample contains too much paraffin, it might not be visible until the sample has cooled down to room temperature after the decrosslinking step. In this case, reheat the sample to 60 °C until the paraffin is visibly melted (maximum 3 minutes). Heating facilitates the transfer of the aqueous (lower) phase. If 3 minutes is not sufficient to melt the paraffin, centrifuge the sample at maximum speed (minimum 19,000 x *g*) for 4 minutes to obtain a more compact interphase. If concerned about further fragmentation due to additional heating, you can skip the heating and just spin the sample for 4 minutes at maximum speed to compress the interphase and make the lower phase more accessible for transfer. See chapter 2.2 for further explanation.

Inefficient removal of paraffin
Sections thicker than 10 µm can result in lower yields due to inefficient removal of paraffin.

RNA yield and concentration is low or **Poor performance in downstream experiments** (continuation)	**Loading of the Spin Column** The correct loading of the Spin Column is crucial for the experimental outcome. Pipet slowly, drop-by-drop, and vertically onto the middle of the Spin Column to not destroy the matrix surface. Do not touch the matrix bed with the pipette tip during sample loading. **Highly tilted matrix in column** A highly tilted matrix after conditioning can lead to insufficient time of interaction with the matrix, which can result in a poor extraction performance. Please read observation “Highly tilted matrix in column” for further instructions.
Air bubbles in Spin Column matrix	**Insufficient homogenization** To homogenize the matrix in the Spin Columns, the Spin Columns need to be vortexed thoroughly. You can perform this step in an upright position, on the side, or upside down depending on what works best for you. If air bubbles are visible, it is mandatory to remove them completely. To remove air bubbles, flick or gently spin down by hand until it is free of air bubbles or quickly vortex again in an upright position at the end. Place each column in a 2 mL reaction tube (not provided) and let them stand to sediment the matrix until used. For improved sedimentation of the matrix, we recommend that this step is performed upon receipt of the kit and to storing them in an upright position. The columns should stand for at least 60 minutes. 

Highly tilted matrix in column	**Inappropriate handling of Spin Column** A highly tilted matrix after conditioning leads to insufficient interaction time between the RNA and the matrix, which can result in poor extraction performance. However, when using fixed-angle rotor centrifuges, the matrix does not become fully horizontal after conditioning. A slightly tilted surface according to the angle of the rotor (usually 30°) is to be expected, and this does not limit the purification ability. But if you observe that the matrix is tilted to a higher angle (see picture below), we recommend prolonging the standing time after resuspending the column up to overnight before conditioning. For quicker processing, we recommend vortexing the Spin Columns upon receipt of the kit and storing them in 2 mL microcentrifuge tubes in an upright position till used. See section 2.2 for further instructions.  Another reason could be that the Spin Column was completely closed during centrifugation and a vacuum was generated. Alternatively, if you want to avoid prolonging the standing time, we suggest using a swing-out rotor centrifuge with our Spin Column Adapter for Plate Centrifuges (product number: 050-011-024)
A_{260}/A_{280} and/or A_{260}/A_{230} value is low	**Wrong blank in measurements** Use supplied Low-TE Buffer (T) as blank. **FFPE sample conditions** Please consider that FFPE samples are highly processed tissues, often resulting in low A_{260}/A_{280} and/or A_{260}/A_{230} values. Use a reference sample or another extraction method to evaluate the results in comparison.

Degraded or fragmented RNA	**FFPE sample conditions** Fragmentation is common for FFPE tissues and can have several reasons like exposure of fresh sample to oxygen, size of the tissue, fixation duration, storage conditions, storage time and more. On top also the decrosslinking of FFPE samples results in higher fragmentation of RNA compared to RNA from fresh samples. This is completely normal. Use a reference sample or another extraction method to evaluate the results in comparison. See section 2.2. for further information on handling FFPE tissues. **Incubation time during decrosslinking** For samples, where a high fragmentation is expected (e.g. very old samples), reducing the 90 °C decrosslinking incubation to 30 minutes can lead to less fragmentation. The incubation time should not be reduced below 30 minutes. However, reducing the incubation time can lead to lower yields.
Occurrence of cross-contamination	**Contaminated pipettes** The use of contaminated pipettes can lead to cross-contaminations. We recommend a separate set of pipettes for sample preparation and PCR preparation, which should be cleaned thoroughly at regular intervals. It is also recommended to use filter tips for all pipetting steps involving samples. **Handling of samples** In general, always work cautiously when handling biological samples. Pay attention to what you're doing and always wear gloves. Keep tubes closed when possible. The use of pipette tips with filters is recommended.

Eluate is missing or volume too low	**Inappropriate handling of Spin Column** The Spin Column was closed during centrifugations, and vacuum was generated. See chapter 2.2 for further instructions on how to handle Spin Columns. **Centrifuge settings** Most centrifuges offer the choice between rpm and *g*-force (rcf); if not, calculate the rpm as described in chapter 2.2. Always make sure to use the correct time mentioned in the protocol to ensure sufficient elution of RNA. **Large amounts of paraffin** An excessive amount of surrounding paraffin can negatively influence the purification process resulting in decreased yield and eluate volume. Therefore, remove as much surrounding paraffin as possible and use only the "usable area" (shown in Figure 1). If your sample contains too much paraffin, it might not be visible until the sample has cooled down to room temperature after the decrosslinking step. In this case, reheat the sample to 60 °C until the paraffin is visibly melted (maximum 3 minutes). Heating facilitates the transfer of the aqueous (lower) phase. If 3 minutes is not sufficient to melt the paraffin, centrifuge the sample at maximum speed (minimum 19,000 x *g*) for 4 minutes to obtain a more compact interphase. If concerned about further fragmentation due to additional heating, you can skip the heating and just spin the sample for 4 minutes at maximum speed to compress the interphase and make the lower phase more accessible for transfer. See chapter 2.2 for further explanation. **Insufficient phase separation** If you observe insufficient separation of phases, centrifuge the sample again at maximum speed and prolong the centrifugation time to 6 minutes. Make sure the centrifuge you are using is set to minimum 19,000 x *g* or go for maximum speed if possible. Also, reheating the sample to remove paraffin may help (see *Large amounts of paraffin* above).

For questions and further troubleshooting, please contact us!

10. LIMITATIONS OF USE

Limitations regarding EchoLUTION FFPE RNA Kit are listed as follows:

- Strict compliance with the user manual is required for successful RNA purification. Following good laboratory practices is crucial for the successful use of the product. Appropriate handling of the reagents is essential to avoid contamination or co-purification of impurities.
- The proof of principle for the EchoLUTION FFPE RNA Kit was evaluated and confirmed using state-of-the-art RT-qPCR and RNA sequencing. Performance parameters are highly dependent on the quality of sample collection.
- This kit is for research use only.

11. SYMBOLS

The following table describes the symbols that appear on the labeling of the EchoLUTION FFPE RNA products and in this user manual.

Table 6: ***EchoLUTION FFPE RNA Kit symbols***

Symbols	Description
	Manufacturer
	For research use only
	Product number
	Batch code
	Contains sufficient for < n > reactions
	Temperature limitation
	Do not re-use
	Expiration date
	Consult instructions for use

WE ARE INTERESTED IN YOUR EXPERIENCE WITH BIOECHO PRODUCTS!

With questions or suggestions or for further troubleshooting, please contact us.

Visit our website and shop for further information, tutorials, and application notes.

This user manual can be found in our shop on the respective product page.

Interested in publishing an application note with us? Please get in touch!

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