RNA Extraction from Decalcified and Non-decalcified Formalin-Fixed Paraffin-Embedded Tissues

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13.1 Introduction and Purpose

This protocol provides a method for obtaining RNA suitable for RT-PCR analysis from decalcified and non-decalcified formalin-fixed and paraffin-embedded (FFPE) tissue specimens. Decalcification causes RNA fragmentation and it is generally believed that this damage precludes successful RT-PCR. For the best performance in calcified tissue, it is necessary to choose an appropriate protocol to decalcify the tissue in order to perform histological, immunological, and molecular studies. The protocol below describes the decalcification procedure using formic acid and nitric acid [1]. For decalcified and non-decalcified FFPE tissue samples, we have standardized an RNA extraction protocol based on a commercial kit (i.e., Pinpoint slide RNA isolation systemII, Zymo Research Corp) [2–4]. This kit for nucleic acid isolation is specifically developed for archive tissues. Some modifications have been made in order to obtain good-quality RNA, depending on the type, volume, and treatment of the tissue [5].

The RNA extraction procedure is based mainly on deparaffinization, tissue isolation, and proteolytic digestion with Proteinase K. The time required for the entire procedure is approximately 6 h.

13.2 Protocol

13.2.1 Reagents

Listed below are the products we use, but similar reagents from other manufacturers may be selected:

- *Nitric acid 65% solution* (Fluka or Sigma-Aldrich)
- *Formic acid 98% solution* (Fluka or Sigma-Aldrich)

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- *Xylene* (Fluka or Sigma-Aldrich)
- *Absolute, 96% and 70% ethanol* (Fluka or Sigma-Aldrich)
- *20 mg/ml Proteinase K (stock solution)*: (Zymo Research Corp). Dissolve 5 mg of Proteinase K in 250 µl of storage glycerol Buffer. Store at −20°C.[1]
- *RNAse Away*[2] (Invitrogen) ready-to-use solution for eliminating RNase and DNA contamination from labware
- *Sterile H_2O*

13.2.2 Equipment

- *Disinfected adjustable pipettes*,[3] range: 0.1–2 µl, 2–10 µl, 20–200 µl, 100–1,000 µl
- *Nuclease-free aerosol-resistant pipette tips*
- *1.5 ml tubes* (autoclaved)
- *Sterile or disposable tweezers*
- *Microtome, with new blade*
- *Orbital shaker*
- *Refrigerated MicroCentrifuge* suitable for centrifugation of 1.5 ml tubes
- *Waterbath*
- *Spectrophotometer*
- *Clean Micro slides with ground edges* (76×26 mm)
- *Glass bowl*
- *Rack for slides*

13.2.3 Method

13.2.3.1 Bone Fixation and Bone Decalcification Protocol

- For a large bone specimen, cut the sample in a large coronal or axial section using the band saw.[4]
- Fix the calcified sections in 4% neutral buffered formalin[5] at room temperature for 24–48 h (for biopsy specimens the time of fixation is approximately 6 h)
- Submerge the samples in the decalcifying solution[6] at room temperature for 7–10 days depending on size and calcification level (for biopsy specimens, the decalcification time is about 1–3 h)
- Wash the samples in tap water to remove the decalcifying solution.
- Dissect the specimen into smaller samples, then proceed with routine paraffin-embedding tissue protocol.

13.2.3.2 Sample Preparation

- If possible, cool the paraffin blocks at −20°C or on ice in order to facilitate cutting of the sections.[7]
- Using a clean, sharp microtome blade,[8] cut two to five sections of 6–8 µm thickness depending on the size of the sample. Discard the first section and displace the other sections on a floating-out bath of clean deionized water at 40°C.
- Mount the sections on clean glass slides using sterile tweezers.
- Dry the sections at 60°C for 10 min.
- The first sections are used for RNA extraction; while the last section is hematoxylin-eosin-stained and used to check the morphology in order to select the area with adequate cellularity and to compare it with the unstained sections.
- Mark the selected area of interest for RNA isolation with a diamond tip.

[1] The solubilization of Proteinase K in glycerol Buffer keeps the solution fluid at −20°C, with a better preservation of the enzymatic activity.

[2] Apply RNAse Away over the surface of glassware or plasticware to be treated. Unwanted RNase and DNA contamination are eliminated.

[3] Clean the pipettes with RNAse Away or leave them under the UV lamp for about 10 min to prevent contaminations. Alternatively, the pipette can be autoclaved, if possible, according to the manufacturer's specifications.

[4] This step should be performed by two operators under a fume hood.

[5] Avoid breathing fumes when working with formalin. The wasted formalin must be collected in a chemical waste container and discharged according to the local hazardous chemical disposal procedures.

[6] For 1 l of decalcifying solution, mix 51ml of 98% formic acid (5% final concentration), 30.8ml of 65% nitric acid (2% final concentration), and 918.2 ml of distilled water. Formic acid and nitric acid are corrosive solutions. Wear gloves and handle the solutions under the chemical hood.

[7] Wear gloves when isolating and handling RNA or reagents for RNA isolation to minimize the contamination with exogenous RNAses. Use autoclaved pipette tips and 1.5 ml microcentrifuge tubes.

[8] Clean the microtome and blade with RNAse Away. It is recommended that the blade be changed after the cutting of each paraffin block to avoid any potential contamination.

13.2.3.3 Deparaffinization

- Submerge the slides in clean xylene at room temperature for 1 h changing the xylene once after approximately 30 min[9].
- Hydrate the slides by washing progressively for 2 min in clean ethanol 100%, 96%, 75%, and then in pure water[10].
- When the tissue has been decalcified, incubate the hydrated slides in 0.001N EDTA pH 8.0 for 40 min in orbital shaker at room temperature.
- Wash the slides in distilled water for 5 min. Repeat this step.
- Air dry the sections, leaving the slide at room temperature for approximately 10 min or under a hood for 2–3 min.

13.2.3.4 Tissue Isolation and Digestion

- Apply the Pinpoint solution to the selected area on the slide to remove the tissue region. The amount of solution depends on the tissue area[11]
- Allow the Pinpoint solution to dry completely at room temperature.[12] When it is dry, it appears as a blue film embedding the tissue and cells underneath.
- Remove the embedded tissue from the slide[13] and transfer to a sterile tube.
- Centrifuge briefly to locate the tissue sample at the bottom of the tube
- Add to the recovered tissue, 25 µl of digestion buffer 1× supplemented with Proteinase K at a final concentration of 1 mg/ml and mix gently. The amount of digestion buffer depends on the amount of tissue. The digestion buffer must cover the tissue pellet completely.
- Incubate the tube in a waterbath at 55°C for 4 h[14].
- Centrifuge the tube briefly at the end of incubation.

13.2.3.5 RNA Extraction

- Add 50 µl of RNA extraction Buffer and mix well with a micropipette.
- Add 75 µl of 100% ethanol to the tube. Vortex lightly.
- Transfer the mixture to a Column in a 2 ml collection tube[15]
- Spin the column at 10,000 rpm in a microcentrifuge for 1 min.
- Add 200 µl of RNA wash buffer to the column and centrifuge at 10,000 rpm for 1 min. Repeat this step.
- Transfer the column into a new RNase-free 1.5 ml tube.
- Add 10 µl of distilled water directly to the membrane. Wait for 2 min.
- Spin the column at 10,000 rpm in a microcentrifuge for 1 min to elute the RNA[16]
- For RNA measurement, pipette 121 µl of sterile water into a fresh tube and add 4 µl of RNA extract (dilution factor = 25). Determine the RNA concentration photometrically at 260 and 280 nm (see Chap. 16, Sect. 16.2.1).[17]

13.2.4 Troubleshooting

- *RNA degradation*. RNA is highly susceptible to RNase digestion, we encourage the use of freshly prepared sections. If a sample cannot be processed immediately, store it at ≤70°C or submerge it in 96% ethanol at −20°C. Processing of tissue sections stored for 1 month or more at room temperature is not recommended.

[9] Avoid breathing fumes when working with xylene. It is better to perform the deparaffinization step under a chemical hood. Xylene is harmful; the wasted xylene must be collected in a chemical waste container and discharged according to the local hazardous chemical disposal procedures.

[10] Make sure that ethanol dilutions used to RNA isolation procedure, are performed in RNase-free water. This step is performed in an orbital shaker (shaking moderately).

[11] Use a sterile pipette tip or a glass pasteur to gently spread a small amount of Pinpoint solution over the selected tissue region. Generally, about 0.5 µl of Pinpoint solution is used per mm^2 of tissue area. Usually, one drop of Pinpoint is adequate for 25 mm^2 of tissue area. Four drops on tissue with appropriate cellularity (using one to four slides) allow good results.

[12] Leave the slides for about 30–45 min; if left under the chemical hood, 10–15 min are sufficient.

[13] Use a sterile blade or scalpel to cut and remove the embedded section from the slide. Transfer the sample to a 1.5 ml tube.

[14] Vortex the tube every 30 min to improve the digestion.

[15] Use one Column for each extraction tube.

[16] The isolated RNA can be used directly for RT-PCR amplification, or it can be stored at −70°C for future use.

[17] The concentration of RNA expressed in µg/µl is obtained as follows: [RNA] = A260×dilution factor×40×10^{-3} (see Chap. 16). An appropriate RNA preparation from FFPE tissue should have a A260/A280 ratio of 1.40–1.80. The ratio variability is linked to the presence of proteins and oligo-, polysaccharides.

- *Insufficient RNA*. Make sure an appropriate sampling area is selected for processing. Select an area of the tissue that will contain ≥50 cells. Increase the sampling area if the tissue type contains few cells (e.g., fatty tissue, fibrous or cartilaginous tissue). The sampling size can vary from 1 mm^2 to over 100 mm^2.
- *DNA contamination*. Traces of fragmented DNA may be present in the eluted RNA fraction. DNA-free RNA can be obtained with subsequent DNase I treatment.

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