

Chapter 2

An Optimized RNA Extraction Method from Archival Formalin-Fixed Paraffin-Embedded Tissue

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Abstract

Formalin-fixed and paraffin-embedded (FFPE) tissue is one of the most valuable resources available for molecular biological analysis on tissue after diagnostic histopathological examination. Gene expression profiles of FFPE can provide insights into molecular mechanisms of disease but are difficult due to the high level of cross-linking of biomolecules by formalin fixation. Despite advances in molecular technologies, the quality of RNA obtained from FFPE tissue remains variable. We have optimized a reliable RNA extraction method for FFPE tissue. This approach is based on deparaffinization at high temperature coupled with a 3-day lysis at 65°C. The average total RNA yield is 4.5–5.5 ng per 1 mm³ of archival FFPE tissue and 260/280 ratios are between 1.80 and 1.95. The extracted RNA has a modal fragment length between 100 and 200 nucleotides by bioanalyzer analysis. Although modal lengths of RNA fragments were shorter, reverse transcription and polymerase chain reaction was able to amplify amplicons in the range of 300 base pairs. This optimized method improves the utility of FFPE tissue for molecular profiling studies.

Key words: Formalin-fixed, paraffin-embedded, tissue, RNA isolation, RT-PCR.

1. Introduction

Gene profiling of formalin-fixed, paraffin-embedded (FFPE) specimens is a powerful tool for biomarker discovery in translational research. However, the recovery of RNA from archival FFPE tissues is challenging because RNA from FFPE tissue suffers from strand breakage and cross-linking by formalin fixation. Cross-linking not only complicates isolation of nucleic acid but also inhibits polymerase during PCR-based molecular assay. To overcome these problems, a number of alternative fixatives and approaches have been examined and discussed (1). Unfortunately,

all alternative fixation methods have limitations either in diagnostic histomorphologic interpretation or in other molecular assays such as immunohistochemistry (1).

Despite excellent RNA extraction methods, RNA quality from FFPE specimens remains variable. RNA isolated from FFPE tissue samples was first described in 1988 by Rupp and Locker (2). Previous studies on RNA extraction have reported varying degrees of success but have used relatively large amounts of tissue for a limited number of specific genes or relatively abundant genes (3–7). No consensus isolation method exists and the data on optimization of protocols are limited. Currently, a limited number of groups routinely isolate RNA from FFPE tissue, and all acknowledge that there is extensive degradation by the formalin fixation process and extraction methodologies are poorly optimized (6). It is therefore imperative that more robust methods of extraction of RNA from FFPE tissue be developed that are compatible and integrated with the current methods used in pathology laboratories around the world.

Currently RT-PCR-based assays are performed on FFPE tissue; however, they require extensive optimization and rely on short amplicons. The introduction of real-time RT-PCR has overcome some of the difficulties of analyzing short RNAs (8, 9). However, the quality of the results of real-time RT-PCR using FFPE tissue depends critically upon the quality and quantity of extracted RNA. For expression profiling to reach its potential in clinical care, the reduction to application on FFPE tissue is essential. Many new methods have been developed and evaluated to address this challenge, with varying degrees of success. In this context, we have established a reliable RNA extraction methodology for archival FFPE tissue (10, 11). The recovery of the RNA is linked to the deparaffinization process, and the capacity to extract the RNA from the paraffin-impregnated tissue. As a fundamental molecular tool, we believe that this new RNA protocol can provide reliable starting material for gene profiling studies via array or RT-PCR-based methodologies.

2. Materials

2.1. Deparaffinization

1. Aqueous dewaxer reagent (AutoDewaxerTM; OpenBiosystem, Huntsville, AL, USA) (*see Note 1*).
2. AutoalcoholTM (OpenBiosystem).
3. Microcentrifuge tubes.
4. Disposable safety scalpels (Miltex Inc., Bethpage, NY, USA).
5. ThermomixerTM (Eppendorf, Westbury, NY, USA).

2.2. RNA Extraction

1. Disposable pellet mixers and cordless motor (VWR, West Chester, PA, USA).
2. Stock solution of RNA lysis buffer: 4 M guanidine isothiocyanate, 25 mM sodium acetate, 0.5% sodium lauryl sarcosinate, pH 5.5 (*see Note 2*). Store at room temperature.
3. Working solution of RNA lysis buffer: Add 10 μ L 14.4 M β -mercaptoethanol per 1 mL lysis buffer before use. The lysis buffer is stable for 1 month after addition of β -mercaptoethanol (*see Note 2*).
4. ThermomixerTM (Eppendorf).
5. Phenol–chloroform–isoamyl alcohol (PCI): Combine phenol (pH 4.3), chloroform, and isoamyl alcohol in the ratio of 25:24:1 (v/v/v) (*see Note 3*).
6. Chloroform–isoamyl alcohol (CI): Combine chloroform and isoamyl alcohol in the ratio of 49:1 (v/v) (*see Note 3*).
7. 3 M sodium acetate (pH 5.2) (Quality Biological Inc., Gaithersburg, MD, USA).
8. 100 and 75% ethyl alcohol (Warner-Graham, Cockeysville, MD, USA).
9. DEPC-treated DW: DEPC (Di-ethyl-pyro-carbonate)-treated water (Quality Biological Inc.).
10. RNase ZAPTM wipes (Ambion, Austin, TX, USA).

2.3. Assessment of the Quality and Quantity of Total RNA

1. NanoDropTM ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).
2. Agilent 2100 BioanalyzerTM (Agilent Technologies, Palo Alto, CA, USA).
3. RNA 600 LabChipTM kit (Agilent Technologies).

2.4. cDNA Synthesis and RT-PCR

1. Random primers (Promega, Madison, WI, USA).
2. SuperScriptTM II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).
3. dNTP Mix (10 mM each, Invitrogen).
4. 0.1 M DTT (Invitrogen).
5. RNasinTM (Promega).
6. RNase H (Invitrogen).
7. PCR SuperMixTM (Invitrogen).
8. Specific primers (sense and antisense).
9. DNase (Promega).
10. PCR tubes (Applied Biosystems, Foster City, CA, USA).
11. PCR machine 9700 (Applied Biosystems).

12. ReadyAgaroseTM Mini Gel: 1% agarose with ethidium bromide (Bio-Rad, Hercules, CA, USA) (*see Note 4*).
13. Ready-loadTM 100 bp DNA ladder (Invitrogen).
14. 1 × Tris acetate EDTA (TAE) buffer: 242 g Tris base, 57.1 mL concentrated glacial acetic acid, 100 mL 0.5 M EDTA, pH 8.0.
15. Alpha-Innotech imaging system (Alpha Innotech Corporation, San Leandro, CA, USA).

3. Methods

The ability to isolate nucleic acid from archived tissue samples provides a powerful molecular analysis tool in retrospective studies of diseased tissue. This method is not novel; rather the approach is based on empiric optimization of existing protocols. The basic protocols are largely derived from other protocols, typically histochemical protocols for staining tissue, and those for the recovery of RNA from fresh or frozen tissue. Our methodology has demonstrated that the deparaffinization step is a significant issue in RNA recovery from FFPE tissue and remains the least optimized step in the RNA extraction process (10). In addition, the RNA yield of our refined methodology was two to threefold higher compared with previous studies and allowed a high purity and quality of RNA (10). This protocol was designed based on two sections of 10-μm thickness archival FFPE tissue specimen (*see Note 5*).

3.1. Deparaffinization

1. Cut two 10-μm sections from FFPE tissue blocks using a microtome. Place the tissue sections in a microcentrifuge tube (*see Note 6*).
2. Add 1 mL of AutoDewaxerTM to the sample and vortex briefly to mix.
3. Centrifuge briefly to bring the tissue that is stuck to the sides of the tube down into the AutoDewaxerTM.
4. Incubate the tube for 15 min at 95°C with vigorous mixing condition (800 rpm) using the ThermomixerTM.
5. Centrifuge the sample for 1 min at room temperature with maximum speed in a microcentrifuge. Remove carefully the AutoDewaxerTM solution without disturbing the tissue pellet. Repeat three times from Steps 2 to 5.
6. Add 1 mL of AutoAlcoholTM to the sample and centrifuge the tube for 1 min at room temperature with maximum speed in a microcentrifuge. Remove carefully the AutoAlcoholTM solution without disturbing the tissue pellet.
7. Dry the sample for 5 min at room temperature (*see Note 7*).

3.2. RNA Extraction

1. Add 300 μL of RNA lysis buffer to the deparaffinized tissue. Homogenize immediately using the disposable pellet mixers and cordless motor until the sample is uniformly homogeneous.
2. Add an additional 300 μL of RNA lysis buffer (final to 600 μL) to the sample. Vortex thoroughly for 1 min to get homogeneous solution (*see Note 8*).
3. Incubate the reaction tube for 72 h at 60°C with mild mixing condition (500 rpm) using the ThermomixerTM.
4. Add 600 μL of equilibrated PCI to the tube. Vortex vigorously for 30 s.
5. Centrifuge the tube at 16,000*g* for 10 min at 4°C in a microcentrifuge. Transfer as much as is easily possible of the upper, aqueous phase to a fresh tube (*see Note 9*).
6. Add an equal volume of CI to the tube. Vortex vigorously for 30 s.
7. Centrifuge the tube at 16,000*g* for 10 min at 4°C and then transfer the upper aqueous phase to a fresh tube.
8. Add 0.1 volume of 3 M sodium acetate buffer (pH 5.2) and 3.0 volume of 100% chilled ethanol. Mix well and allow the RNA to precipitate at -20°C overnight.
9. Centrifuge at 16,000*g* for 30 min at 4°C. Discard the supernatant.
10. Add 1 mL of 75% chilled ethanol to the pellet and then centrifuge the tube at 16,000*g* for 10 min at 4°C. Discard the supernatant.
11. Dry the precipitated RNA for 5–10 min at room temperature.
12. Dissolve the RNA in 20 μL of DEPC-treated distilled water (*see Note 10*).

3.3. Assessment of the Quality and Quantity of Total RNA

1. Take 1 μL of the sample to measure the concentration and quality of the RNA.
2. Turn on the NanoDropTM ND-1000 Spectrophotometer and set up a reference using blank solution (DEPC-treated DW).
3. Apply 1 μL of the RNA to the NanodropTM and press a measure button (*see Note 11*).
4. To check the quantity and quality of the RNA sample (*see Note 12*), take 150–200 ng of the RNA sample and apply to Agilent 2100 BioanalyzerTM. An example of the results produced is shown in **Fig. 2.1**.

3.4. cDNA Synthesis and RT-PCR

Synthesize the first-strand cDNAs from the isolated total RNAs using reverse transcriptase and random primers.

1. Anneal 8 μL (2 μg) total RNA templates with 1 μL (500 ng) random primer in a sterile RNase-free microcentrifuge tube.

3.4.1. Synthesis of First-Strand cDNA

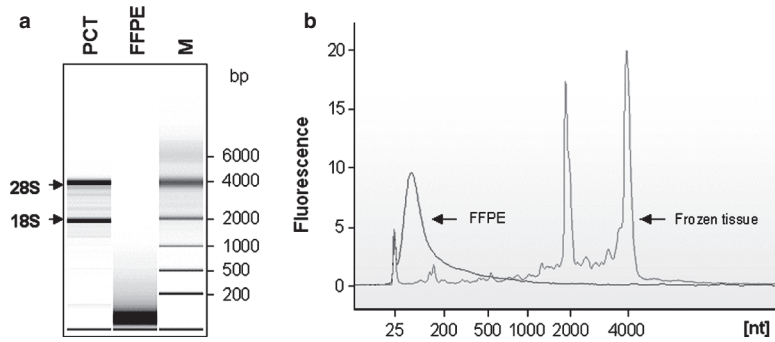


Fig. 2.1. The profiling of total RNA extracted from archival formalin-fixed and paraffin-embedded (FFPE) tissue by microcapillary electrophoresis. We analyzed RNA quality using the Agilent 2100 BioanalyzerTM, using 200 ng of total RNA extracted from rat kidney FFPE tissues. Representative data were presented as a gel-like image (a) and an electropherogram (b). PCT: positive control (frozen rat kidney); FFPE: formalin-fixed and paraffin-embedded tissue; M: RNA 6000 nano ladder (Agilent Technologies).

2. Heat the tube at 70°C for 5 min and allow it to slowly cool to room temperature to finish annealing. Briefly spin down the mixture to the bottom.
3. To the annealed primer/template, add the following in the order shown. Gently spin well after each addition.
 - a. First-strand 5 × buffer (4 µL)
 - b. 0.1 M DTT (2 µL)
 - c. 10 mM dNTP mix (2.5 mM each) (2 µL)
 - d. RNasinTM (1 µL)
 - e. SuperScriptTM II (2 µL)
4. Incubate the tube at 42°C for 2 h and place on ice (*see* **Note 13**).
5. Inactivate the reaction by heating at 70°C for 15 min and store the tube on ice for 2 min. Briefly spin down the mixture to the bottom.
6. Add 1 µL (two units) of RNase H and briefly spin down the mixture. Incubate the tube at 37°C for 20 min.
7. Add 80 µL of RNase-free water to the mixture and store at –20°C until amplification by PCR.

3.4.2. cDNA Amplification

Amplify the specific cDNA of interest by PCR using specific primers.

1. In a sterile RNase-free microcentrifuge tube on ice, add the following in the listed order for amplification of one sample:
 - a. PCR SuperMix (45 µL)
 - b. Molecular-grade distilled water (3 µL)
 - c. Template cDNA (1 µL) (*see* **Note 14**)
 - d. Sense primer (200 µM) (0.5 µL)
 - e. Antisense primer (200 µM) (0.5 µL)

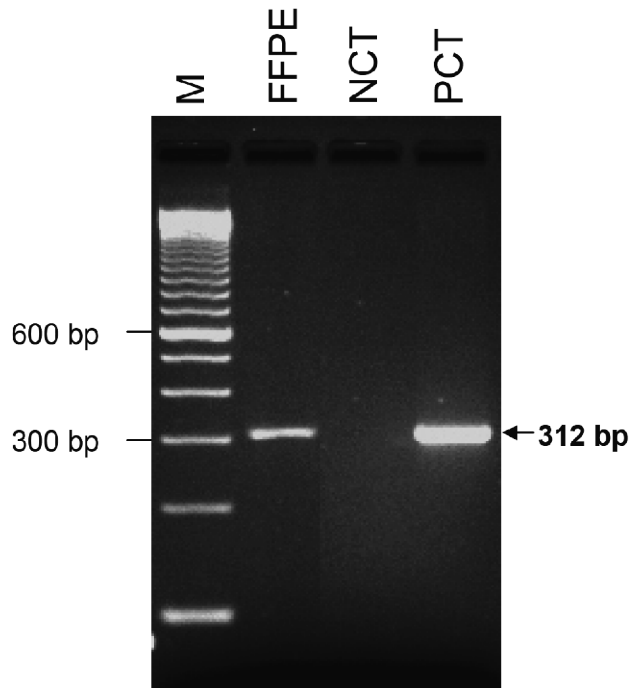


Fig. 2.2. Expression of rat *GAPDH* by RT-PCR analysis in total RNA derived from formalin-fixed paraffin-embedded (FFPE) kidney tissue. cDNA was synthesized using a random hexamer, and amplicons were separated on a 1% ready agarose gel (Bio-Rad). Positive as well as negative controls had been included in the amplification experiments. FFPE: formalin-fixed and paraffin-embedded tissue; NCT: negative control (without cDNA); PCT: positive control (fresh rat kidney); M: ready-loadTM 100 bp DNA ladder (Invitrogen).

2. Carry out PCR amplification for 35 cycles in the PCR machine 9700.
3. To confirm the size of PCR amplicons, carry out agarose electrophoresis using 1% ReadyAgaroseTM Mini Gel with $1 \times$ TAE buffer. An example of the results produced is shown in Fig. 2.2.

4. Notes

1. The aqueous-based dewaxer was as efficient as xylene and allowed the use of higher temperatures than xylene for the deparaffinization steps.
2. 4 M guanidine isothiocyanate and β -mercaptoethanol are strong RNase inhibitors. The RNA lysis buffer is toxic. Wear gloves, a laboratory coat, and eye protection when preparing, handling, or working with solution.

3. These reagents are toxic and should be used in the fume hood. Gloves should be worn when working with these reagents. The remnant of these reagents should be decanted into a special container. Do not discard into sink.
4. EtBr (ethidium bromide) is a neurotoxin. Gloves should always be worn when working with the reagents. The used EtBr solution must be collected in a special container and disposed of in accordance with guidelines. Stock solutions of EtBr should be stored in light-tight bottles at 4°C.
5. This method has been optimized for whole tissue sections. Application to micro-dissected sections has not been validated. Issues with reference to micro-dissected specimens include volume of material as well as previous preparative steps of deparaffinization and staining. Exposure to aqueous states during staining is associated with RNA degradation. Additionally, it should not be assumed that the deparaffinization steps for micro-dissection are sufficient for optimal RNA isolation, and additional deparaffinization should be carried out after micro-dissection.
6. If you are using archival FFPE slide specimens, carefully collect tissue in the microcentrifuge tube using a disposable safety scalpel.
7. The pellet should become white.
8. To minimize contamination by DNA trapped at the interface, avoid taking the lowest part of the aqueous phase.
9. As long as the sample is in RNA lysis buffer, RNase may be inhibited by guanidinium thiocyanate.
10. DEPC is a carcinogen and should be handled with care. Gloves should be worn when handling the reagent.
11. The NanoDropTM can automatically and simultaneously measure RNA at wavelengths of 260 and 280 nm, and calculate and display RNA concentration together with the ratio of A_{260}/A_{280} reading numbers on the computer screen.
12. The extracted RNA should have a ratio of 1.80 to 1.95 of A_{260}/A_{280} reading numbers. The concentration of the RNA sample is variable depending on FFPE tissue quality.
13. At this stage, the synthesis of the first-strand cDNA is completed and safe for RNase contamination.
14. PCR experiments demonstrate that amplicons up to 300 bp are feasible; however, to achieve these, the investigator must use more cDNA template than required for the same amplicons using RNA from fresh tissues.

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