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Extraction of DNA and RNA from Formalin-fixed Paraffin-embedded Tissue Specimens

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[Abstract] One of the most common methods for isolating biomolecules from clinical samples is using TRIzol. While many tissues are freshly frozen, a large number of tissues are formalin-fixed paraffin-embedded (FFPE) blocks for pathology and diagnostics. Isolation of biomolecules (DNA and RNA) from FFPE blocks can be a tedious task since extensive protein-nucleic acid and protein-protein crosslinking heavily modifies nucleic acids. Recently, many kit-based protocols have made it convenient to isolate small biomolecules from FFPE blocks. The protocol reported here is adapted from the Qiagen FFPE DNA isolation kit and the TRIzol method for RNA isolation and optimized. We believe that this optimization will help to improve DNA and RNA yield.

Keywords: Formalin-fixed paraffin-embedded tissues, DNA, RNA, TRIzol, Biomolecule extraction

[Background] In recent years, next-generation sequencing (NGS) has provided seamless benefits in the fields of medical diagnostics, oncology, and precision medicine (Kerick *et al.*, 2011). While NGS has enabled better therapeutic approaches, the identification of a particular mutation causing a disease and the course of action for treatment remedies needs substantial validation (Arreaza *et al.*, 2016). An ideal and sensitive approach is to validate the candidate genes as biomarkers using RT-qPCR, for which a good quality starting material in the form of biomolecules (DNA/RNA) is required. However, the extraction procedure using samples from various collection and preservation methods has been a great challenge, especially formalin-fixed paraffin-embedded (FFPE) tissue blocks (Heydt *et al.*, 2014). FFPE tissue blocks are one of the most common methods of tissue preservation since they allow preservation of cellular components and architecture (Donczo and Guttman, 2018; Groelz *et al.*, 2018), can provide specimen quickly, and are essential in the histopathology lab for diagnosis. The disadvantage of this method is that longer exposure to formalin may cause crosslinking of proteins and nucleic acids, which can result in potential DNA/RNA damage (Williams *et al.*, 1999; Do and Dobrovic, 2015, Atanesyan, 2017). Previously, FFPE samples were not considered a good source for molecular analysis, since nucleic acids are extensively modified in this process (Blow *et al.*, 2007; Bass *et al.*, 2014). Steps such as preparation, fixation, embedding, and storage can affect DNA/RNA quality, which can further influence later stage diagnostics and research (Nam *et al.*, 2014; Turashvili *et al.*, 2012). In the recent past, efforts have been made to isolate smaller quantities/aliquots of DNA/RNA from FFPE samples using appropriate proteinase digestion and buffers (Sah *et al.*, 2013, Sarnecka *et al.*, 2019). The purified

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biomolecules could be used for further downstream processes such as polymerase chain reaction (PCR), quantitative reverse transcription PCR (RT-qPCR), microarray, and next-generation sequencing analyses (Taga *et al.*, 2013; Ludyga *et al.*, 2012; Gupta *et al.*, 2020). Although commercial kits are available for FFPE extraction, there is a need for a better protocol describing extraction procedures for biomolecules (DNA/RNA) from FFPE tissue specimens. This protocol is mainly based on the deparaffinization of FFPE blocks and proteolytic digestion with proteinase K/protease. We have further optimized this protocol for DNA and RNA extraction using a QIAamp FFPE DNA extraction kit and the TRIzol method, respectively.

Part I: Protocol for DNA isolation

Materials and Reagents

1. Nuclease-free pipette tips (Axygen)
2. 1.5-ml autoclaved Eppendorf tubes (Tarson, catalog number: 500010)
3. 2-ml autoclaved Eppendorf tubes (Tarson, catalog number: 500020)
4. Xylene Extrapure AR, 99.8% (SRL, catalog number: 90998)
5. Absolute ethanol (Merck, catalog number: 100983)
6. RNase A (Sigma, catalog number: 10109134001)
7. Agarose (Sigma, catalog number: A9539)
8. QIAamp FFPE tissue kit (Qiagen, catalog number: 56404)
9. 1 kb DNA ladder (NEB, catalog number-N3232S)
10. 100 bp DNA ladder (NEB, catalog number-N3231S)
11. Isopropyl alcohol/2-propanol (Merck, catalog number: 107022)
12. 70% ethanol (see Recipes)
13. 80% isopropyl alcohol (see Recipes)

Equipment

1. Coplin jars
2. Sterile pipettes: 0.5-10 µl, 2-20 µl, 20-200 µl, 100-1,000 µl (Eppendorf)
3. Sterile forceps/tweezers
4. Centrifuge at room temp (Thermo Scientific, model: Heraeus Pico 21)
5. Centrifuge at 4°C (Thermo Scientific, model: Heraeus Biofuge Primo R)
6. Heat block (Techne Dri-Block DB-3D)
7. Thermomixer C (Eppendorf)
8. NanoDrop™ (Thermo Scientific, catalog number: ND-1000)
9. Qubit 3.0 Fluorometer (Life Technologies, Invitrogen, catalog number: Q33216)
10. Vortex mixer (Labnet, model: VX 100)

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11. Electrophoresis unit
    a. Mini-Sub Cell GT Horizontal Electrophoresis System and PowerPac Basic Power Supply (Bio-Rad, catalog number: 1640300)
    b. Sub-Cell GT Horizontal Electrophoresis System, 15 × 15 cm tray, with casting gates (Bio-Rad, catalog number: 1704402)
12. Gel Doc™ XR+ Systems (Bio-Rad, catalog number: 170-8195)

Procedure

A. Sample preparation

Cut 2-10 sections at 5-µm thickness using microtome or scalpel blades. Place the sections, weighing approximately 50-100 mg each (of one sample), in a 1.5-ml nuclease-free microcentrifuge tube using sterile forceps.

B. Deparaffinization

1. Add 1 ml xylene per sample (sample weight: approximately 50-100 mg) and vortex vigorously for 10 s.
2. Place the tubes in a heat block at 50°C for approximately 30-40 min.
   Note: This step is modified from the original QIAamp protocol. This step is required for complete removal of paraffin from the samples as paraffin might interfere with the isolation process. Increasing the deparaffinization time (usually it takes around 10-24 h) gives better DNA yield. The ultimate goal is to remove all paraffin from the sections. Depending on the section size, the deparaffinization time can be adjusted.
3. Centrifuge at 14,000 × *g* (12,000 rpm) for 1 min at room temperature (15-25°C). Carefully remove the xylene using a 1-ml pipette without disturbing the pellet.
4. Add 1 ml absolute ethanol, vortex, and centrifuge at 14,000 × *g* (12,000 rpm) at room temperature for 1 min. Carefully discard the supernatant without disturbing the pellet and repeat the ethanol wash one more time.
5. Allow the tubes to air dry at room temperature for about 30-45 min.
   Note: Alternative method for deparaffinization. The following steps are also modified from the original QIAamp protocol. This removes paraffin more efficiently and gives a better DNA yield.
6. Alternatively, for deparaffinization, we can take 2-10 sections at 5-µm thickness (weighing approximately 50-100 mg) on a glass slide placed on a Metallic Slide Stand with the section side facing upward. Incubate the glass slide in a hot air oven at 100°C for 30 min.
7. After incubation, place the glass slides in a Coplin jar filled with xylene for 5 min. Repeat this step one more time.
8. Place the glass slide in 80% isopropyl alcohol (usually glass slides are placed in a Coplin jar filled with alcohol) for 3 min. Then place the slide to 100% isopropyl alcohol (usually glass slides are placed in a Coplin jar filled with alcohol) for 5 min.

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9. Scrape the tissues off the slide into a 1.5-ml nuclease-free microcentrifuge tube using a sterile scalpel blade.
10. Add 1 ml xylene per sample and vortex vigorously for 10 s.
11. Centrifuge at 14,000 × *g* (12,000 rpm) for 5 min at room temperature. Carefully discard the supernatant without disturbing the pellet. Repeat this step two more times.
12. Add 1 ml absolute ethanol, vortex, and centrifuge at 14,000 × *g* (12,000 rpm) for 5 min at room temperature. Carefully discard the supernatant and repeat the ethanol wash one more time.
13. Allow the tubes to air dry at room temperature for about 30-45 min.

C. DNA extraction

1. All procedures were carried out according to the manufacturer's instructions (QIAamp FFPE tissue kit).
2. Add 180 µl ATL buffer (lysis buffer) with proteinase K.
3. Incubate the samples in a thermomixer overnight (18-24 h) at 55°C, shaking moderately (around 800-900 rpm) for complete lysis.
   Notes:
   a. *This step is modified from the original QIAamp method.*
   b. *This step results in complete lysis of the sample and can be further increased to 48 h depending on the sample size and thickness.*
4. Incubate at 90°C in a thermomixer/heat block for 1 h.
5. Briefly centrifuge the tubes at 6,000 × *g* (8,000 rpm) at room temperature to remove drops from inside the lid.
6. Allow the tubes to reach room temperature, add 2 µl RNase A (100 mg/ml), and incubate for 2 min at room temperature.
   Note: This step is required to obtain RNA-free genomic DNA.
7. Add 200 µl AL buffer (Lysis/Binding buffer) and vortex thoroughly. Add 200 µl absolute ethanol and vortex again. To obtain a homogenous solution, it is essential to mix AL buffer and ethanol immediately; these can also be premixed to save time.
8. Briefly centrifuge the tubes at 6,000 × *g* (8,000 rpm) at room temperature to remove drops from inside the lid.
9. Transfer the entire lysate to the QIAampMinElute column with a 2-ml collection tube and centrifuge at 6,000 × *g* (8,000 rpm) at room temperature for 1 min. Discard the flowthrough.
10. Add 500 µl AW1 buffer (wash buffer) and centrifuge at 6,000 × *g* (8,000 rpm) at room temperature for 1 min. Discard the flowthrough.
11. Add 500 µl AW2 buffer (wash buffer) and centrifuge at 6,000 × *g* (8,000 rpm) at room temperature for 1 min. Discard the flowthrough.
12. Centrifuge at full speed (20,000 × *g*; 14,000 rpm) at room temperature for 3 min to dry the membrane. Place the column into a new 1.5-ml Eppendorf tube (elution tube).

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13. Add 50-70 µl ATE buffer (Elution buffer) directly onto the membrane. Incubate for 5 min at room temperature.
    Note: Incubating the eluate column with ATE buffer for 5 min gives a better DNA yield.
14. Centrifuge at full speed (20,000 × *g*; 14,000 rpm) at room temperature for 1 min and collect the eluate.
15. The overall extraction procedure is pictorially represented in Figure 1.

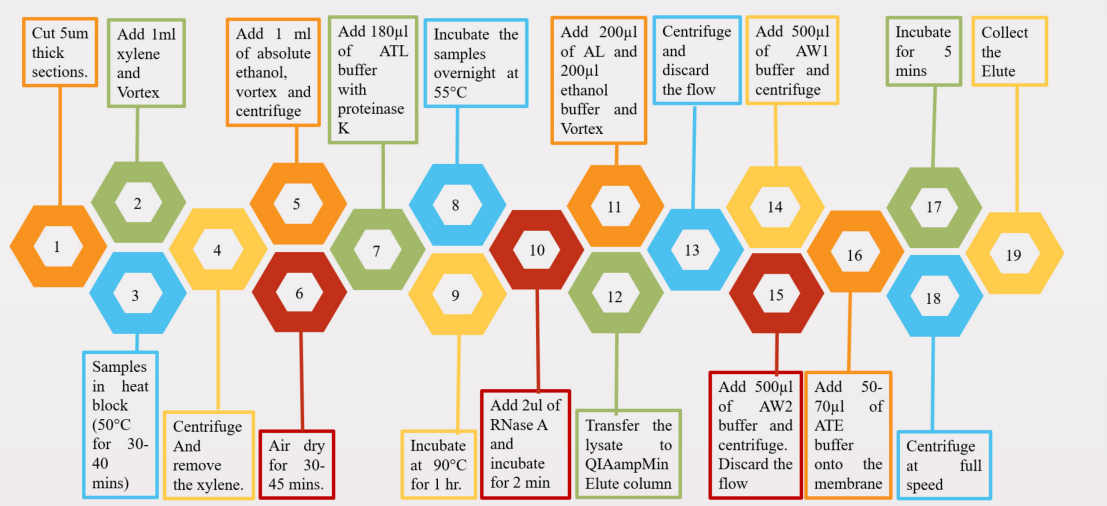

Figure 1. Flowchart for the steps involved in DNA isolation from FFPE tissues

16. DNA Quantitation: The DNA concentration was determined using two different methods: spectrophotometric measurement (NanoDrop-ND1000, Thermo Scientific) and fluorometric measurement (Qubit 3.0 Fluorometer/Life Technologies, Invitrogen). The concentration and absorbance values obtained by both methods are listed in Table 1.

Table 1. DNA concentration and purity using the original Qiagen and modified protocols

	Qiagen original		Qiagen modified	
Sample No.	**Concentration (ng/µl)**	**Absorbance (260/280)**	**Concentration (ng/µl)**	**Absorbance (260/280)**
1	35	2.19	83.4	1.81
2	50.9	2.02	64.4	1.83
3	21.8	1.89	36.9	1.79
4	46.4	1.9	63.4	1.82
5	52.8	2.02	61.9	1.81
6	20.8	1.96	66.6	1.83
			113.8	1.82
			142.6	1.85
			299	1.86

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D. Quality check

The DNA quality was examined using the absorbance ratio of 260 nm to 280 nm (A_{260}/A_{280}). DNA has a maximum absorbance (optical density, OD) at 260 nm, and the conversion factor for DNA concentration is A_{260} ssRNA = 50 µg/ml. Samples that fell between 1.8 and 2.0 were considered good quality. A lower ratio indicates the presence of contaminants in the sample. The samples were also assessed by 1.5% agarose gel electrophoresis (Figure 2).

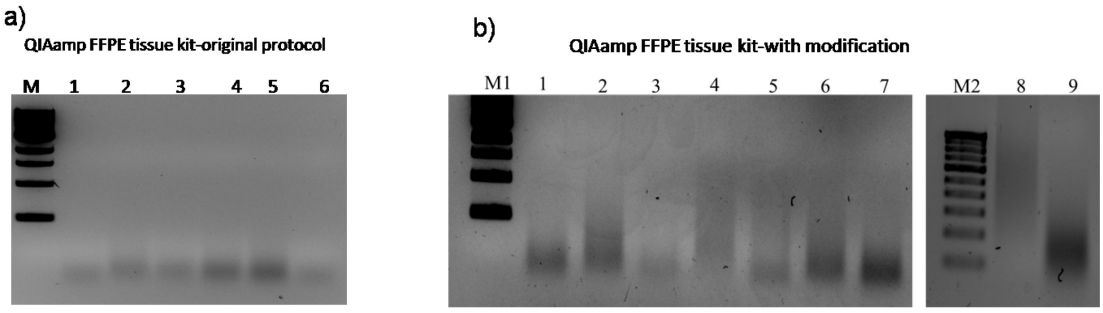

Figure 2. Agarose gel electrophoresis of isolated DNA from FFPE blocks. a) DNA isolation using the original Qiagen protocol: M, 1Kb ladder; lanes 1-6, malignant prostate cancer FFPE specimens. b) DNA isolation using the modified Qiagen protocol: M1, 1Kb ladder; M2: 100 bp ladder, and lanes 1-9, isolated DNA from malignant prostate cancer FFPE specimens (Figure 1).

Part II: Protocol for RNA isolation

Materials and Reagents

1. Sterile pipettes: 0.5-10 µl, 2-20 µl, 20-200 µl, 100-1,000 µl (Eppendorf)
2. Nuclease-free pipette tips (Axygen)
3. 1.5-ml autoclaved Eppendorf tubes (Tarson, catalog number: 500010)
4. Xylene Extrapure AR, 99.8% (SRL, catalog number: 90998)
5. Absolute ethanol (Merck, catalog number: 100983)
6. TRIzol (Invitrogen, catalog number: 15596018)
7. Tris-HCl (Sigma-Aldrich, catalog number: T6066-1KG)
8. SDS (Sigma-Aldrich, catalog number: L3771-1KG)
9. Calcium chloride dihydrate (Merck, catalog number: 02382)
10. Protease (20 mg/ml) (Qiagen, catalog number: 1043368)
11. Chloroform (Merck, catalog number: 102445)
12. 2-propanol (Merck, catalog number: 107022)
13. RNase-free H_2O or DEPC-treated H_2O (see Recipes)
14. Digestion buffer (see Recipes)

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Equipment

1. Sterile forceps/tweezers
2. Centrifuge at room temp (Thermo Scientific, model: Heraeus Pico 21)
3. Centrifuge at 4°C (Thermo Scientific, model: Heraeus Biofuge Primo R)
4. Heat block (Techne Dri-Block DB-3D)
5. Thermomixer C (Eppendorf)
6. NanoDrop™ (Thermo Scientific, catalog number: ND-1000)
7. Gel Doc™ XR+ Systems (Bio-Rad, catalogue number: 170-8195)
8. Electrophoresis unit
   a. Mini-Sub Cell GT Horizontal Electrophoresis System and PowerPac Basic Power Supply (Bio-Rad, catalog number: 1640300)
   b. Sub-Cell GT Horizontal Electrophoresis System, 15 × 15 cm tray, with casting gates (Bio-Rad, catalog number: 1704402)

Procedure

A. Sample preparation

Cut 2-10 sections at 5-µm thickness using microtome or scalpel blades. Place all the sections (weighing approximately 100 mg) in a 1.5-ml Eppendorf tube using sterile forceps.

B. Deparaffinization

1. Add 1ml xylene per sample and vortex vigorously for 10 s.
2. Place the tubes in a heat block at 50°C for approximately 30-40 min.
   Note: This step is modified from the original protocol. This step is required for complete removal of paraffin from the samples that might interfere with the later isolation steps. Increasing the deparaffinization time (usually takes around 10-24 h) gives a better RNA yield. The ultimate goal is to remove all paraffin from the blocks. Depending on the sample size, the deparaffinization time can be adjusted.
3. Centrifuge at 14,000 × *g* (12,000 rpm) at room temperature for 1 min and carefully discard the supernatant.
4. Add 1 ml absolute ethanol, vortex, and centrifuge at 14,000 × *g* (12,000 rpm) at room temperature for 1 min. Discard the supernatant and repeat the ethanol wash one more time.
5. Allow the tubes to air dry at room temperature for about 30-45 min.

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C. RNA extraction

1. Add 150-200 µl digestion buffer per sample supplemented with 500 µg/ml proteinase K. The amount of digestion buffer depends on the sample size (weight/volume ratio). In this protocol, we used 200 µl digestion buffer per 100 mg sample.
   Note: For up to 100 mg tissue, 200 µl digestion buffer is sufficient, but if we want to increase the sample amount, the volume of digestion buffer needs to be increased accordingly.
2. Incubate the samples in a thermomixer overnight (18-24 h) at 55°C, shaking moderately (around 800-900 rpm).
   Notes:
   a. *This step is modified from the original TRIzol method.*
   b. *This step results in complete lysis of the sample. This step can be further increased to 48 h depending on the sample size.*
3. Add 1 ml TRIzol per sample. Incubate at 25-30°C for 10 min to allow the nucleoprotein complexes to dissociate.
4. Add 0.2 ml chloroform per sample, vortex for 15-20 s, and incubate at 25-30°C for 5 min in a heat block.
5. Centrifuge at 12,000 × *g* (10,000 rpm) for 15 min at 4°C. The mixture separates into a lower organic phase, an interphase, and an upper aqueous phase. Transfer the aqueous phase to a new autoclaved 1.5-ml tube.
   Note: Be extra careful at this step to avoid transferring the protein-containing interphase.
6. Add 0.6 ml 2-propanol per sample for precipitation. Place at -20°C for 1 h.
   Note: Tubes can be kept overnight.
7. Centrifuge at 12,000 × *g* (10,000 rpm) at 4°C for 10 min and discard the supernatant.
8. Wash the pellet with 1 ml 70% ethanol. Carefully discard the supernatant without disturbing the pellet.
9. Air dry the pellet and resuspend in an appropriate amount of RNase-free or DEPC-treated water.
10. Determine the RNA concentration using the NanoDrop.
11. Store the RNA at -70°C.
12. The overall extraction procedure is pictorially represented in Figure 3.

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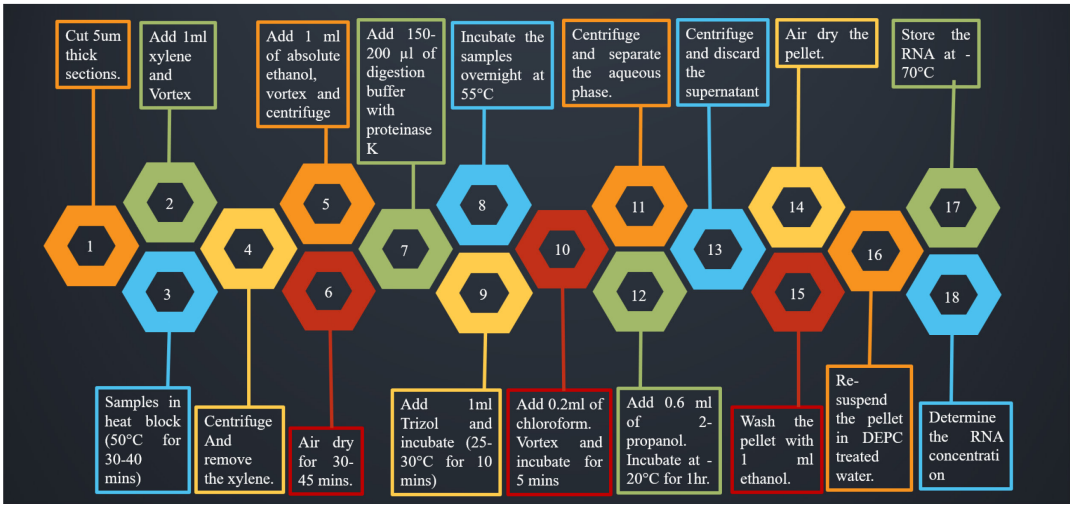

Figure 3. Flow chart for the steps involved in RNA isolation from FFPE tissues

13. RNA Quantitation: The RNA concentration was determined using spectrophotometric measurement (NanoDrop-ND1000, Thermo scientific). The program in the spectrophotometer automatically reads the OD at 230, 260, and 280 nm and calculates the different ratios described below. The concentration and absorbance values are listed in Table 2.

Table 2. RNA concentration and purity using the original and modified TRIzol protocols

	TRIzol original		TRIzol modified	
Sample No.	**Concentration (ng/µl)**	**Absorbance (260/280)**	**Concentration (ng/µl)**	**Absorbance (260/280)**
1	31.2	1.58	250.2	2.05
2	121.3	1.63	240.7	1.98
3	61.8	1.55	280.4	2.03
4	49.8	1.73	253.8	1.89
5	66.1	1.67	290.8	1.97
6	259.2	1.77	300	2.05

D. Quality check

The RNA quality was examined using an absorbance ratio of 260 nm to 280 nm (A_{260}/A_{280}). RNA has a maximum absorbance (optical density, OD) at 260 nm, and the conversion factor for RNA concentration is A_{260} ssRNA = 40 µg/ml. Samples with an A_{260}/A_{280} value around 2.0 were considered good quality. A lower ratio is indicative of the presence of protein contaminants. A high peak at A_{230} indicates the presence of phenol, guanidine, or reagents other than protein contamination. Further, RNA integrity can be checked by running the samples on a 1.5-2.0% agarose gel (in 1× TAE buffer) (Figure 4). Another possible method for checking RNA integrity is using a Bioanalyzer (https://www.agilent.com/cs/library/applications/5989-1165EN.pdf) that measures the size of ribosomal RNA (rRNA) bands and assesses the RNA quality according to an RNA integrity number (RIN).

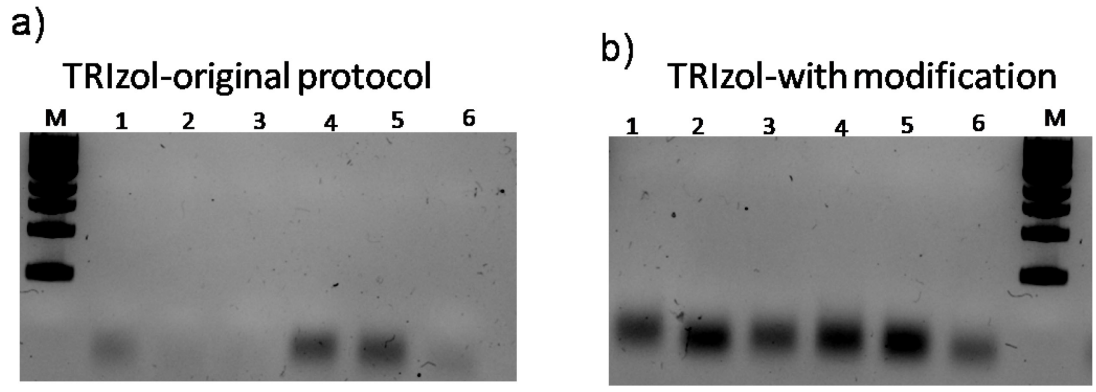

Figure 4. Agarose gel electrophoresis of RNA isolated from FFPE blocks. a) RNA isolation using the original TRIzol protocol. b) RNA isolation using the modified TRIzol protocol. M, 1Kb ladder; lanes 1-6, malignant prostate cancer FFPE tissues.

Conclusions: We present a protocol that describes DNA and RNA extraction using the Qiagen/TRIzol method, respectively, with slight modifications. We observed that making these modifications significantly improved the DNA/RNA quality and quantity. We found that keeping the sections in xylene for a longer time results in the proper removal of paraffin, and increasing the lysis time increases the DNA/RNA yield. We observed that the DNA concentration measured using a NanoDrop was higher than that seen by Qubit analysis, which could be due to contamination from other impurities present in the samples (Nakayama *et al.*, 2016). Interestingly, quantitative analysis of DNA extracted from non-FFPE tissues shows a concordance between spectrophotometric and fluorometric methods; therefore, it can be concluded that the observed differences between the NanoDrop and Qubit readings may be a result of FFPE tissue processing (O'Neill *et al.*, 2011). Moreover, incomplete deparaffinization is one of the key factors determining the quantitative evaluation of isolated DNA/RNA from FFPE tissues. Thus, this protocol gives the important steps and their modifications to improve DNA/RNA quality and quantity from FFPE tissues.

Notes

A. Important points to consider before starting the isolation
   1. Incubating the eluate column with ATE buffer for 5 min gives a better DNA yield.
   2. Lysis steps can be further increased to 48 h for both DNA and RNA isolation, but proteinase K should be added intermittently.
   3. Extreme precaution should be taken when working with RNA, since RNA is less stable than DNA and is prone to contamination with RNases present in the environment. To avoid this, wipe all the work spaces with 70% ethanol and wear gloves during all isolation steps.
   4. Store the FFPE blocks at room temperature, which makes it easier to cut the sections.
   5. Be very careful when making the sections; sometimes it can be difficult to scrape off the wax.

B. The same strategies apply more or less with changed compositions for QIAzol and TwistBio extraction methods.

Recipes

1. Digestion buffer for RNA isolation
   20 mM Tris-HCl (pH-8.0)
   0.5% Sodium Dodecyl Sulphate (SDS)
   1 mM $CaCl_2$
2. DEPC-treated water for RNA isolation
   Add 1 ml diethyl pyrocarbonate (DEPC, to a final concentration of 0.1%) to 1 L sterile water Incubate at 37°C overnight and autoclave.
3. Ethanol 70%
   To prepare 100 ml 70% ethanol, add 30 ml sterile (DNase/RNase-free) water to 70 ml absolute ethanol and mix well.
4. Isopropyl alcohol 80%
   To prepare 100 ml 80% isopropyl alcohol, add 20 ml sterile (DNase/RNase-free) water to 80 ml absolute isopropyl alcohol and mix well.

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Author's contributions: Nidhi Shukla and Narmadhaa Siva standardized the protocol with the help of suggestions from Krishna Mohan Medicherla, Madhu Balaji, and Prashanth Suravajhala. Nidhi Shukla wrote the first draft of the manuscript. Avadh Shah, Rabia Parveen, and Ashwani Kumar Mishra added an image for the extraction of DNA/RNA QC. PS and KM proofread the manuscript before all authors agreed to the changes.

Competing interests

The authors declare no competing interests associated with this work.

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