DNA Extraction from Formalin-Fixed Paraffin-Embedded (FFPE) Tissues

7

Serena Bonin, Patricia J.T.A. Groenen, Iris Halbwedl, and Helmut H. Popper

Contents

7.1 Introduction and Purpose

This protocol provides a method of obtaining DNA suitable for PCR analyses from formalin-fixed and paraffin-embedded (FFPE) tissue specimens [1–4], even of autopsy origin [5, 6]. This procedure is based mainly on deparaffinization of tissues (as optional step) and a proteolytic digestion with Proteinase K. The proteolysis step is fundamental to degrade proteins and generate pure DNA. The time required for the whole procedure is 4 days.

Commercial kits are also available for DNA extraction from FFPE; some of these are specifically dedicated to archive tissues (i.e., QIAamp DNA FFPE Tissue Kit). However, some minor modifications could be useful to achieve better results depending on the type of tissues; requirements for the specific molecular test should be taken into consideration [7].

7.2 Protocol

7.2.1 Reagents

Note: Reagents from specific companies are reported here, but similar reagents from other providers could be used:

- *Xylene* (Fluka or Sigma-Aldrich)
- *Absolute, 90% and 70% Ethanol* (Sigma)
- *20 mg/ml Proteinase K* (stock solution): (Sigma P2303) Dissolve 100 mg of Proteinase K in 5 ml of autoclaved 50% glycerol diluted in sterile H_2O.[1]

[1]The solubilization of Proteinase K in 50% sterile glycerol maintains the solution fluid at −20°C with a better preservation of the enzymatic activity.

S. Bonin (✉)
Department of Medical, Surgical and Health Sciences, University of Trieste, Cattinara Hospital, Strada di Fiume 447, Trieste, Italy

P.J.T.A. Groenen
Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

I. Halbwedl and H.H. Popper
Research Unit for Molecular Lung and Pleura Pathology, Institute of Pathology, Medical University of Graz, Graz, Austria

G. Stanta (ed.), *Guidelines for Molecular Analysis in Archive Tissues*,
DOI: 10.1007/978-3-642-17890-0_7,

Store at −20°C. The stock solution of 20 mg/ml should be diluted to a final concentration of 1 mg/ml Proteinase K in digestion buffer

- *10× Digestion Buffer*[2] (stock solution): 500 mM Tris HCl pH 7.5, 10 mM EDTA, 1 M NaCl, 5% Tween 20
- *1× Digestion Buffer*: 50 mM Tris HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.5% Tween 20. Complete the solution with Proteinase K (1 mg/ml = final concentration) just before use
- *Phenol-Tris buffered pH 8*[3]*/$CHCl_3$ 50:50*: Mix 1 part of buffered phenol with 1 part of chloroform. Top the organic phase with 1× TE buffer (about 1 cm height) and allow the phase to separate. Store at 4°C in a light-tight bottle
- *Phenol-Tris buffered pH 8/$CHCl_3$–isoamyl alcohol 50:49:1*: Mix 48 ml of Chloroform with 2 ml of isoamyl alcohol. Mix 1 part of buffered phenol with 1 part of chloroform–isoamyl alcohol. Top the organic phase with 1× TE buffer (about 1 cm high) and allow the phase to separate. Store at 4°C in a light-tight bottle
- *Iso-propanol or EtOH/Sodium acetate*
- *1 mg/ml Glycogen in water*
- *10× TE buffer*: 100 mM Tris pH 8, 10 mM EDTA pH 8

7.2.2 Equipment

- *Disinfected*[4] *adjustable pipettes*, range: 2–20 µl, 20–200 µl, 100–1,000 µl
- *Nuclease-free aerosol-resistant pipette tips*
- *1.5 ml tubes* (autoclaved)
- *Single-packed toothpicks*
- *Sterile or disposable tweezers*
- *Microtome, with new blade*
- *Centrifuge* suitable for centrifugation of 1.5 ml tubes at 13,200 or 14,000 rpm
- *Thermoblock*
- *Thermomixer* (e.g., Eppendorf)
- *SpectroPhotometer*

7.2.3 Method

7.2.3.1 Sample Preparation

- If possible, cool the paraffin blocks at −20°C or on dry ice in aluminium foil in order to cut the sections.
- Using a clean, sharp microtome blade[5], cut two to ten sections of 5–10 µm thickness depending on the size of the sample. Discard the first section and displace the other ones in 1.5 ml tubes, using a sterile toothpick or tweezers (depending on the section size). Use some sections from a paraffin block without included tissue, treated together with other samples, for negative control analysis.

7.2.3.2 Deparaffinization[6] (Optional)

- Add 1 ml of xylene,[7] vortex for 10," and then maintain the tube at room temperature for approximately 5 min.[8]
- Spin the tube for 5 min at maximum speed (14,000 rpm) in a microcentrifuge and then carefully remove and discard the supernatant using a micropipette or a glass Pasteur pipette.[9]
- Repeat wash with a fresh aliquot of xylene.

[2]It is possible to digest the proteins using the following buffer: PCR buffer 1× final (10 mM Tris–HCI pH 8.3, 50 mM KCl) and Proteinase K, 1 mg/ml final. The use of this buffer, without EDTA and detergent, is suggested to avoid the possible inhibition of PCR reaction by the omitted reagents.

[3]We strongly recommend purchase of saturated phenol pH 8 from a commercial manufacturer.

[4]Clean the pipettes with alcohol or another disinfectant and leave them under the UV lamp for 10 min. Alternatively, it is possible to autoclave the pipette depending on the provider instructions.

[5]Clean the microtome with xylene.

[6]Deparaffinization step could be completely skipped; alternatively, it could be performed by adding 300 µl of mineral oil to the tube containing the section and incubating at 90°C for 20 min to dissolve the wax [8].

[7]When working with xylene, avoid breathing fumes. It is better to perform the deparaffinization step under a fume hood.

[8]Wear gloves when isolating and handling DNA to minimize the contamination with exogenous nucleases. Use autoclaved pipette tips and 1.5 ml microcentrifuge tubes.

[9]Xylene is harmful; the wasted xylene must be collected in a chemical waste container and discharged according to the local hazardous chemical disposal procedures.

- Wash the pellet by adding 1 ml of absolute ethanol. Flick the tubes to dislodge the pellet and then vortex the tubes for 10 s.
- Leave at room temperature for approximately 5 min.
- Spin the tube for 5 min at maximum speed (14,000 rpm) in a microcentrifuge and then carefully remove and discard the supernatant.
- Repeat washes using 90% and 70% ethanol.
- After removing 70% ethanol, allow the tissue pellet to air dry in a thermoblock at 37°C for about 30 min.

7.2.3.3 Proteolytic Digestion and DNA Extraction

- Add to the tissue pellet 150–300 µl of digestion buffer 1x supplemented with Proteinase K at final concentration of 1 mg/ml. The amount of digestion buffer depends on the tissue amount. The digestion buffer must cover the tissue pellet completely.[10]
- Incubate in the thermomixer for 48–72 h[11] at 55°C, shaking moderately. For longer digestion, Proteinase K can be added again every 24 h.
- Add 1 volume of phenol[12]-buffered pH 8.0 Tris/$CHCl_3$ /isoamyl alcohol (50:49:1 v/v/v).[13] Mix well by inverting the tube, and leave on ice for 10–20 min.
- Centrifuge at 14,000 rpm at 4°C for 20 min. The mixture will separate into a lower organic phase, an interphase, and an upper aqueous phase.
- Transfer the upper phase into a new tube, add 1 volume of $CHCl_3$, mix well for 5 min, and centrifuge at 14,000 rpm at 4°C for 20 min.
- Transfer the supernatant in a new tube containing 5 µl of glycogen solution (1 mg/ml stock) as precipitation carrier. Carefully avoid transferring the interphase containing proteins.
- Precipitate overnight at –20°C with 1 volume of iso-propanol or 2.5 volumes of EtOH supplemented with 0.1 volumes of Sodium acetate 3 M pH 7.
- Centrifuge at 14,000 rpm at 4°C for 20 min and discard the supernatant.
- Wash the pellet with 200 µl of 70% ethanol without resuspending the pellet to wash away the remaining salts.
- Air dry the pellet and resuspend the DNA pellet in the appropriate amount of TE buffer 1×. Store the DNA solution at –20°C.
- For DNA measurement, pipette 199 µl of sterile water into a fresh tube and add 1 µl of DNA extract (dilution factor = 200). Determine the DNA concentration photometrically at 260 and 280 nm (see Chap. 16, Sect. 16.2.1 for more details).[14]

7.2.4 Troubleshooting

- If the DNA yield is low, you may have lost the DNA pellet; in such case, repeat the entire process of extraction.
- If the pellet is not visible after centrifugation, the precipitation could have been incomplete because of the absence of a precipitation carrier. Add 5 µl of glycogen 1 mg/ml, and leave at –20°C overnight to complete precipitation.
- If DNA is absent, a nuclease contamination could have occurred. In such case, repeat the extraction using freshly made reagents.

[10] If the pellet is firmly lodged at the bottom of the tube, it is possible to dislodge it in the digestion buffer using a sterile toothpick.

[11] Longer digestion time (at least 48 h) increases the yield of the DNA.

[12] Phenol is very toxic and should be handled in a fume hood; the wasted phenol must be collected with hazardous chemical waste.

[13] The extraction can also be performed with 1volume of phenol (Tris saturated)-chloroform-(50:50, v/v). Phenol is an inhibitor of PCR reaction, because of Taq Polymerase inactivation. A single chloroform-isoamyl alcohol (24:1, v/v) extraction could be performed after the phenol (Tris saturated)-chloroform-isoamyl alcohol extraction in order to completely remove phenol traces.

[14] The concentration of dsDNA expressed in µg/µl is obtained as follows: $[DNA] = A_{260} \times$ dilution factor $\times 50 \times 10^{-3}$ (see Chap. 16). A clean DNA preparation should have a A_{260}/A_{280} ratio of 1.5–2. This ratio is decreased by the presence of proteins, oligo-, and polysaccharides. Concentration estimation can also be affected by phenol contamination, as phenol absorbs strongly at 260 nm and therefore can mimic higher DNA yield and purity.

References

1. Gilbert MT, Haselkorn T, Bunce M, Sanchez JJ, Lucas SB, Jewell LD, Van Marck E, Worobey M (2007) The isolation of nucleic acids from fixed, paraffin-embedded tissues-which methods are useful when? PLoS ONE 2(6):e537
2. Lassmann S, Gerlach UV, Technau-Ihling K, Werner M, Fisch P (2005) Application of BIOMED-2 primers in fixed and decalcified bone marrow biopsies: analysis of immunoglobulin H receptor rearrangements in B-cell non-Hodgkin's lymphomas. J Mol Diagn 7(5):582–591
3. Lehmann U, Kreipe H (2001) Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. Methods 25(4):409–418
4. Pauluzzi P, Bonin S, Gonzalez Inchaurraga MA, Stanta G, Trevisan G (2004) Detection of spirochaetal DNA simultaneously in skin biopsies, peripheral blood and urine from patients with erythema migrans. Acta Derm Venereol 84(2): 106–110
5. Bonin S, Petrera F, Niccolini B, Stanta G (2003) PCR analysis in archival postmortem tissues. Mol Pathol 56(3): 184–186
6. Bonin S, Petrera F, Stanta G (2005) PCR and RT-PCR analysis in archivial postmortem tissues. In: Encyclopedia of diagnostic genomics and proteomics. Marcel Dekker, New York
7. Bonin S, Hlubek F, Benhattar J, Denkert C, Dietel M, Fernandez PL, Hofler G, Kothmaier H, Kruslin B, Mazzanti CM, Perren A, Popper H, Scarpa A, Soares P, Stanta G, Groenen PJ (2010) Multicentre validation study of nucleic acids extraction from FFPE tissues. Virchows Arch 457(3): 309–317
8. Lin J, Kennedy SH, Svarovsky T, Rogers J, Kemnitz JW, Xu A, Zondervan KT (2009) High-quality genomic DNA extraction from formalin-fixed and paraffin-embedded samples deparaffinized using mineral oil. Anal Biochem 395(2): 265–267