RNA Temperature Demodification

14

Serena Bonin and Giorgio Stanta

Contents

14.1 Introduction and Purpose

The extraction of useful RNA from formalin-fixed and paraffin-embedded (FFPE) tissues is often compromised for the extraction efficacy. Moreover, RNA in FFPE is not completely available for reverse transcriptase-polymaerase chain reaction (RT-PCR) reactions because it is resistant to extraction due to cross-linking with proteins [1, 2]. RNA, as DNA, is modified in FFPE tissues by the presence of methylol addition. Prolonged fixations could favour further reactions with the above-mentioned groups, resulting in irreversible artifacts. Masuda et al. [1] demonstrated that all four bases showed addition of mono-methylol (CH_2OH) in formalin, with different rates, ranging from 40% for adenine to 4% for the less reactive uracil. The presence of the methylol group doesn't allow the reverse transcription reaction, but the presence of the majority of these groups could be removed, by simply heating the RNA extracts in formalin-free buffers. Several methods have been reported to demodify RNA obtained from FFPE [1, 3, 4]; here we report two simple temperature treatments derived from Masuda [1] and Li [3]. It is also possible to use more sophisticated methods for RNA demodification, such as the one described by Oberli [4]. The latter is based on a chemical treatment with NH_4Cl, followed by a heating step at 94°C . The starting point of the protocol described hereafter will be RNA extracts from FFPE tissues (See Chap. 12 for more details).

From our experience, by the use of these simple methods it is possible to decrease the Ct values in real-time RT-PCR by about two cycles when compared to untreated RNA extracts. Moreover, the best results are achieved by the use of "Method I" (described below): resuspension of the RNA in TE buffer 1× at pH 7.5. Nevertheless the demodification treatment seems to improve the RNA recruitment only in old samples.

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

G. Stanta
Department of Medical Sciences,
University of Trieste, Cattinara Hospital,
Strada di Fiume 447, Trieste, Italy

G. Stanta (ed.), *Guidelines for Molecular Analysis in Archive Tissues*,
DOI: 10.1007/978-3-642-17890-0_14, © Springer-Verlag Berlin Heidelberg 2011

The methods described hereafter could be added as routine procedure to the RNA extraction protocols especially for very old samples.

14.2 Precautions

Glassware, plasticware, micropipette, and reagents must be kept in specifically dedicated RNase-free areas. Sterile, disposable plasticware should preferably be used. General laboratory glassware or plasticware should be presoaked in 0.1% DEPC-treated H_2O for 2 h at 37°C. The DEPC-treated items should be rinsed thoroughly with DEPC H_2O and then autoclaved. Alternatively, glassware could be put in oven at 250°C for at least 2 h.

After DEPC treatment the water is essentially free from RNases and can be used to prepare solutions or to rinse any items to use for RNA isolation. Every solution for RNA extraction should be treated with 0.1% DEPC or made with DEPC H_2O. Note, however, that DEPC reacts quickly with amines and so it cannot be used to treat solutions containing Tris. Tris buffers should be made using Tris prepared in already DEPC-treated H_2O.

It is better to weigh all the reagents by tapping directly from the bottle on the technical balance covered by a new aluminium foil, without touching the powder with any instrument. Alternatively, it is possible to use stainless steel spatula or spoons that have been previously treated in oven at 250° for at least 2 h.

14.3 Protocol

14.3.1 Reagents

Note: All reagents should be RNase-free or DEPC-treated and used exclusively for RNA analysis.

- *DEPC-treated water (H_2O DEPC)*: Add 1 ml of diethylpirocarbonate (DEPC) (0.1% final concentration) to 1 l of sterile water and incubate overnight at 37°C; then autoclave[1]
- *TE buffer 1× pH 7.5:* 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0). Stock solution 10×. For this purpose it is possible to use both pH 7.5 or pH 8 TE buffers; however, in our experience we obtained better results by using TE buffer pH 7.5
- *TE buffer 10×:* 100 ml 1 M Tris-HCl pH 7.5, 20 ml 500 mM EDTA pH 8.0, 880 ml DEPC water. Autoclave

14.3.2 Equipment

- *Disinfected*[2] *adjustable pipettes*, range: 2–20 µl, 20–200 µl, 100–1,000 µl
- *Nuclease-free aerosol-resistant pipette tips*
- *1.5 or 2 ml tubes* (autoclaved)
- *Thermoblock*

14.3.3 Method I

Note: Derived from Masuda [1] with minor modifications; it can be used in already extracted RNA.

1. RNA pellets from standard extraction procedure[3] (See Chap. 12 for details) should be resuspended in about 20 µl of TE buffer 1× pH 7.5.
2. Incubate in a thermoblock at 70°C for 20 min.[4]
3. Chill on ice.
4. Spin down briefly to collect the drops.
5. Store the RNA solution at –70°C in aliquots[5].
6. For RNA measurement, pipette 199 µl of sterile water into a fresh tube and add 1 µl of RNA extract. Determine the RNA concentration photometrically at 260 and 280 nm[6] (See Chap. 16).

[1]DEPC is a carcinogen and should be handled with care under a fume hood.

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

[3]For this demodification method, it is not mandatory to use the RNA extraction protocol described in this book; any extraction procedure, even commercial kits, can be used.

[4]It is possible to protract this step up to 1 h; however, in our experience, the best results have been obtained by heating at 70°C for 20 min.

[5]It is better to store RNA extracts in small aliquots to prevent multiple thawing/freezing, which may degrade the nucleic acid.

[6]The concentration of RNA expressed in µg/µl is obtained as follows: $[RNA] = A_{260} \times \text{dilution factor} \times 40 \times 10^{-3}$; for example, when diluting 1 µl RNA in 199 µl sterile water, the dilution factor is 200. A clean RNA preparation should have a A_{260}/A_{280} ratio of 1.5–2.0. This ratio is decreased by the presence of proteins, phenol and oligo-, polysaccharides.

14.3.4 Method II [3]

Note: It can be used during RNA extraction procedures using the crude RNA extract after the proteolysis step.

1. Crude RNA extracts (after the proteolysis step) in Proteinase K buffer[7] (See Chap. 12 for details) should be incubated in a thermoblock at 70°C for 20 min.
2. Chill on ice.
3. Spin down briefly to collect the drops on the tube walls and proceed with protocol for RNA extraction to purify RNA extracts.

14.3.5 Troubleshooting

- If the yield of RNA is low by UV measurement decrease the dilution factor to check the concentration and repeat the measuring.
- If RNA is absent, an RNase contamination could have occurred. In such case, it is better to check all the solutions and materials used along the extraction procedure, and repeat the entire process.

[7]For this demodification method, it is not mandatory to use the proteinase K digestion buffer described in this book; any proteinase K buffer could be used, even the ones provided in commercial kits or commercial solutions.

References

1. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K (1999) Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res 27(22): 4436–4443
2. Srinivasan M, Sedmak D, Jewell S (2002) Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 161(6):1961–1971
3. Li J, Smyth P, Cahill S, Denning K, Flavin R, Aherne S, Pirotta M, Guenther SM, O'Leary JJ, Sheils O (2008) Improved RNA quality and Taqman pre-amplification method (preamp) to enhance expression analysis from formalin fixed paraffin embedded (FFPE) materials. BMC Biotechnol 8:10
4. Oberli A, Popovici V, Delorenzi M, Baltzer A, Antonov J, Matthey S, Aebi S, Altermatt HJ, Jaggi R (2008) Expression profiling with RNA from formalin-fixed, paraffin-embedded material. BMC Med Genet 1:9