Optimizing nucleic acid extraction from thyroid fine-needle aspiration cells in stained slides, formalin-fixed/paraffin-embedded tissues, and long-term stored blood samples

Otimização da extração de ácidos nucleicos de material de punção aspirativa por agulha fina de tiroide obtido de lâminas coradas, tecidos fixados em formalina e emblocados em parafina e amostras de sangue estocadas por longo período

Marina M. L. Kizys[1*], Mirian G. Cardoso[1*], Susan C. Lindsey[1], Michelle Y. Harada[1], Fernando A. Soares[1], Maria Clara C. Melo[1], Marlyn Z. Montoya[1], Teresa S. Kasamatsu[1], Ilda S. Kunii[1], Gisele Giannocco[1,2], João Roberto M. Martins[1,3], Janete M. Cerutti[4], Rui M. B. Maciel[1], Magnus R. Dias-da-Silva[1,3]

[1] Laboratory of Molecular and Translational Endocrinology, Department of Medicine, Escola Paulista de Medicina, Universidade Federal de Sao Paulo (Unifesp/EPM), Sao Paulo, SP, Brazil
[2] Department of Morphology and Physiology, Faculdade de Medicina do ABC (FMABC), Santo Andre, SP, Brazil
[3] Laboratory of Molecular and Translational Endocrinology, Department of Biochemistry, Unifesp/EPM, Sao Paulo, SP, Brazil
[4] Laboratory of Molecular and Translational Endocrinology, Department of Morphology, Unifesp/EPM, Sao Paulo, SP, Brazil
* These authors contributed similarly to the study

Correspondence to:
Magnus R. Dias da Silva
Laboratório de Endocrinologia Molecular e Translacional
Rua Pedro de Toledo, 669, 11° andar
Universidade Federal de São Paulo
04039-032 – São Paulo, SP, Brazil
magnus.bioq@epm.br

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ABSTRACT

Objective: Adequate isolation of nucleic acids from peripheral blood, fine-needle aspiration cells in stained slides, and fresh and formalin-fixed/paraffin-embedded tissues is crucial to ensure the success of molecular endocrinology techniques, especially when samples are stored for long periods, or when no other samples can be collected from patients who are lost to follow-up. Here, we evaluate several procedures to improve current methodologies for DNA (salting-out) and RNA isolation. **Materials and methods:** We used proteinase K treatment, heat shock, and other adaptations to increase the amount and quality of the material retrieved from the samples. **Results:** We successfully isolated DNA and RNA from the samples described above, and this material was suitable for PCR, methylation profiling, real-time PCR and DNA sequencing. **Conclusion:** The techniques herein applied to isolate nucleic acids allowed further reliable molecular analyses. Arq Bras Endocrinol Metab. 2012;56(9):618-26

Keywords
DNA; RNA; nucleic acid extraction; FNA; FFPE tissue; blood

RESUMO

Objetivo: O isolamento adequado de ácidos nucleicos a partir de sangue periférico, lâmina corada de punção aspirativa por agulha fina, tecido fixado em formalina e emblocado em parafina e tecido fresco é fundamental para assegurar o sucesso de técnicas aplicadas em endocrinologia molecular, principalmente quando lidamos com amostras estocadas por longos períodos ou quando há impossibilidade de nova coleta de amostra de pacientes que perderam o seguimento. Neste trabalho, objetivamos otimizar as metodologias clássicas para a extração de DNA (salting-out) e RNA. **Materiais e métodos:** Utilizamos proteinase K, choque térmico, dentre outras modificações, com o objetivo de aumentar a quantidade e a qualidade do material recuperado a partir das amostras descritas acima. **Resultados:** Isolamos com sucesso DNA e RNA de tais amostras e o material obtido foi adequado para a realização de PCR, perfil de metilação, PCR em tempo real e sequenciamento de DNA. **Conclusão:** As técnicas aplicadas neste estudo para isolar ácidos nucleicos permitiram a realização posterior de análises moleculares consistentes e confiáveis. Arq Bras Endocrinol Metab. 2012;56(9):618-26

Descritores
DNA; RNA; extração de ácido nucleico; PAAF; tecido FFEP; sangue

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INTRODUCTION

Advances in molecular biology have promoted the routine use of techniques such as PCR, real-time PCR, and DNA sequence analysis for the detection of mutations in a large variety of human diseases (1). In molecular endocrinology, these approaches are of enormous importance for the diagnosis of inherited disease-causing mutations and/or somatic mutations. Therefore, the adequate isolation of nucleic acids from peripheral blood, fine-needle aspiration (FNA) cells in stained slides, and fresh and formalin-fixed/paraffin-embedded (FFPE) tissues is crucial to ensure the success of these techniques, posing a challenge for routine DNA extraction protocols, especially when blood samples and FFPE tissues have been stored for long period, and when no other samples can be collected from patients lost to follow-up (2,3).

Ideally, DNA and RNA isolation methods should maximize nucleic acid yield, and isolated material should be usable in all downstream molecular applications (3). However, some extraction methods used in commercial kits have relatively poor recovery of DNA from blood samples stored longer than 3 months, for example. Moreover, lab protocols based exclusively on commercial kits are generally insufficient to promptly overcome these limitations.

DNA and RNA isolated from cells in stained slides are very important due to possible molecular testing in conjunction with cytology, especially when the latter reveals indeterminate lesions (4). Furthermore, obtaining DNA and RNA from FFPE samples and stained slides is always a challenge because fixation, embedding, staining, and extraction methods generally inhibit nucleic acid retrieval from the samples (3). On the other hand, although formalin fixation may degrade nucleic acids, it also deactivates nucleases and, thus, has a stabilizing effect (3).

Although a number of studies have reported promising results in optimizing commercial kits using automated or manual DNA extraction methods, little has been published about the optimization of in-house nucleic acid extraction methods. Here, we report adaptations carried out in procedures to obtain greater amount of DNA and RNA of better quality extracted from several sources. Because our research is focused on thyroid disorders, including thyroid cancer, we used PCR, real-time PCR, methylation profiling, and direct sequencing analyses of the *RET*, *BRAF* and *HES1* genes as examples to demonstrate and validate such improvements.

METHODS AND RESULTS

DNA isolation from fresh and long-term stored blood samples, FFPE tissue samples, *FNA cells in stained* slides and cultured cells

Fresh whole blood

We extracted genomic DNA using a method modified from Bowtell (5). A 300-µL fresh whole-blood sample was mixed with 900 µL of buffer A (20 mM Tris-HCl pH 7.6) to promote red blood cell lysis for 10 min at room temperature (RT). After centrifugation for 1 min at 15,600 x*g* at RT, the supernatant was removed, and the pellet was resuspended in 600 µL of cold buffer B (10 nM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.1% w/v SDS), which contains denaturing agents for cell lysis. The pellet was then pipetted up and down until it was completely dissolved. Next, the sample was submitted to heat shock (incubation at -70°C for 20 min followed by 66°C for 20 min), followed by treatment with 5 µL of proteinase K (10 mg/ml) at 66°C for 1 hour and at RT for 15 min. For protein precipitation, 200 µL of solution C were added (5M potassium acetate, 11.5% glacial acetic acid), and the sample was then vortexed vigorously for 20 s and centrifuged for 5 min at 15,600 x*g*. The supernatant was transferred to a clean microcentrifuge tube and mixed with 600 µL of isopropanol and, when necessary, 1 µL of glycogen (20 mg/µL), as well, mixed by manual inversion. After centrifugation at 15,600 x*g* for 2 min, a DNA-glycogen pellet was observed, and the supernatant was removed. After the pellet was washed with 70% ethanol, centrifuged, and air-dried, it was resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). Resuspension can be optimized by incubation at 65°C for 60 min, or overnight. DNA extraction can also be enhanced by blood subfractionation, and selection of the leukocyte layer (the buffy coat after 10-min centrifugation at 2,880 x*g*).

If the first nucleic acid extract was still rich in contaminants (protein or lipids), a phenol-chloroform-isoamyl re-extraction was performed. After centrifugation with phenol-chloroform, the aqueous phase containing the nucleic acid was re-extracted with equal volumes of chloroform-isoamyl alcohol, followed by a series of 100% and 70% ethanol washes.

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Long-term stored blood

For DNA extraction from blood samples that were stored for long periods, we modified several steps of the process, such as the use of three incubation periods with buffer A (to promote red cell lysis). Moreover, a longer incubation time for the heat shock is also necessary (2 hours at both temperatures), as well as an overnight incubation with proteinase K at 66°C. Figure 1 shows DNA isolated from long-term stored blood compared with DNA isolated from fresh blood samples.

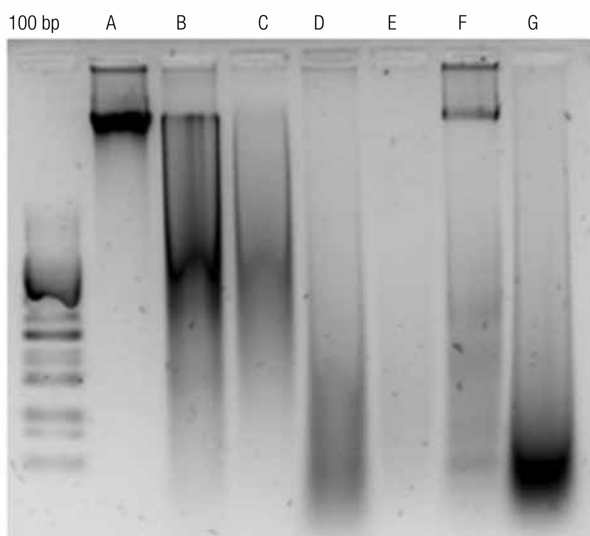

Figure 1. Agarose gel (1.8%) electrophoresis of total DNA isolated from fresh blood (**A**), long-term stored blood (**B**), fresh tissue (**C**), FFPE tissue (**D**), bisulfite treated DNA from FFPE tissue (**E**), B104 cells (**F**), and FNA cells in stained slides (**G**). DNA concentration and quality were evaluated using Nanodrop2000 (Thermo Scientific, Wilmington, DE, USA) to measure absorbance at 260 and 280 nm. Compared with DNA recovered from fresh blood (lane A), long-term stored blood seems to be more degraded and produces smeared bands (lane B). DNA recovered from FFPE tissue (lane D) appears as a light smear, and the DNA concentration is lower than that obtained from fresh and long-term stored blood, as expected. In lane E, DNA smears are even lighter after bisulfite treatment. Minor smearing can be noted in DNA recovered from cells (lane F), although less extensive than that observed for long-term stored blood, FFPE tissue and bisulfite-treated DNA. Furthermore, DNA isolated from cells and fresh blood exhibited greater concentration and quality, suggesting little degradation and preserved structure. Most of the DNA isolated from FNA cells in stained slides, unlike DNA isolated from other samples, migrated to the bottom of the electrophoresis gel (lane G).

Formalin-fixed/paraffin-embedded tissues

For FFPE tumor tissues, we performed some adaptations to enhance the yield of recovered DNA. For each sample, one 10-µm tissue slice was deparaffinized by means of two xylene (1 mL) baths at 65°C for 30 min with periodic inversion, followed by centrifugation for 5 min at 15,600 x*g* and removal of the supernatant. The pellet was washed with 100% ethanol and subsequently with 70% ethanol, combined with incubation for 1 min at RT, followed by centrifugation for 5 min at 15,600 x*g*, and finally air-dried at 37°C. For cell lysis, we added 600 µL of cold buffer B and 2.5 µL of proteinase K (20 mg/mL), mixed by inversion, and incubated the mixture for 2 hours at 56°C. Proteinase K was inactivated by incubating the sample at 95°C for 10 min and transferring it to ice for 1 min. For DNA purification, we used 200 µL of solution C, inverted the sample and centrifuged it for 5 min at 15,600 x*g*. The supernatant was transferred to a clean microcentrifuge tube and submitted to the steps described above for genomic DNA. Results are shown in figure 1, and compared with samples treated with commercial kit in figure 2.

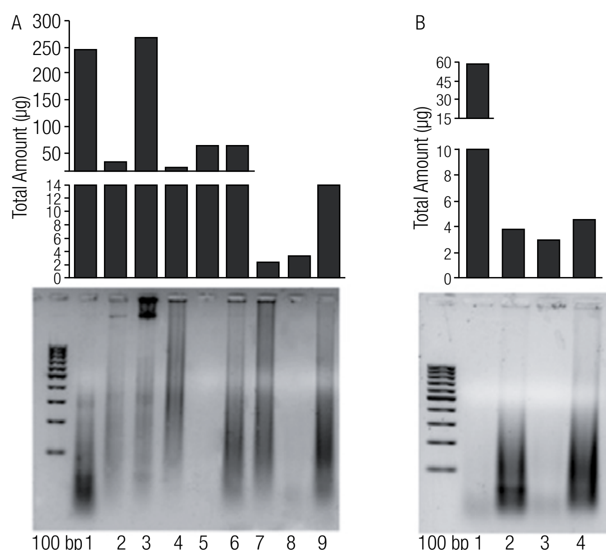

Figure 2. Representative histogram showing the total amount of DNA directly measured by spectrophotometry (upper panel) and correlated DNA quality analysis in 1.8% electrophoresis agarose gel (bottom panel) after loading 400 ng of DNA from each different source per lane. A: DNA isolated from B104 cell line (lanes 1-3), FFPE tissue (lanes 4-6) and FNA cells in stained slides (lanes 7-9) using three tested protocols; commercial kit (lanes 1, 4 and 7), TRIzol™ RNA/DNA standard co-extraction (lanes 2, 5, and 8), and in-house modified salting-out (lanes 3, 6, and 9). B: Total DNA obtained from FFPE tissue (lanes 1 and 2) and FNA cells in stained slides (lanes 3 and 4), using TRIzol™ RNA/DNA standard co-extraction (lanes 1 and 3) and TRIzol™ RNA/DNA in-house optimized co-extraction (lane 2 and 4).

FNA cells in stained slides

DNA extraction from thyroid FNA stained slides followed the in-house protocol; however, the method was modified by resuspending the cells in 300 µL of cold buffer B, pipetting up and down and scraping, followed by transfer to a clean microcentrifuge tube. Next, 1.5 µL of proteinase K (10 mg/mL) were added, mixed by

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inversion, and incubated at 55°C for at least 2 hours (up to overnight). Proteinase K was inactivated at 95°C for 10 min, and the sample was incubated on ice for 1 min. To precipitate the protein in the sample, 100 µL of solution C were added, and the sample was vortexed for 20 s, incubated for 5 min on ice and centrifuged for 3 min at 15,600 x*g*. The supernatant was transferred to a clean microcentrifuge tube, mixed with 300 µL of isopropanol and 0.5 µL of glycogen (20 mg/µL) by inversion, and centrifuged for 5 min at 15,600 x*g*. The next steps were the same used for genomic DNA extraction described above.

Cultured cells

To isolate DNA from cells in culture (1-2x10^6 to 1-2x10^7), neither buffer A nor proteinase K are necessary. To improve the protocol, we removed the growth medium from the culture dish, washed the cells with 1 mL 1xPBS to remove residual medium and added 1 mL 1xPBS to detach the cells. For adherent cells, we first treated the culture with 1 mL trypsin for 5 min at 37°C, and then inactivated trypsin by adding 3 mL of medium. After transferring the cells (resuspended in PBS) to a labeled microcentrifuge tube, we centrifuged the sample for 30 s at 15,600 x*g* and removed the supernatant, leaving approximately 20 µL of residual liquid. The pellet was then resuspended by vortexing. Buffer B (300 µl) was added, and the sample was pipetted up and down to completely lyse the cells, followed by the addition of 100 µL of buffer C. The tube was inverted and centrifuged for 1 min at 15,600 x*g*. The supernatant was transferred to a clean microcentrifuge tube and submitted to the steps described above for genomic DNA, but with only half the volume of isopropanol and ethanol.

RNA isolation from blood, fresh and FFPE tumor tissue, *FNA cells in stained slides* and cell lines

Fresh whole blood

Samples of 3 mL of peripheral whole blood were collected from patients in tubes with EDTA. For each 250 µL of peripheral blood collected, 750 µL of TRIzol LS™ reagent (Life Technologies, Carlsbad, CA, USA) was added to a clean microcentrifuge tube. During this step, it is important to avoid touching the tube wall to prevent the formation of clots, which decreases the extraction yield. The sample was homogenized by vortexing for 10 s (this must be performed immediately after adding the blood to prevent clot formation) and incubated for 5 min at RT. For each 750 µL of TRIzol LS™ reagent used for homogenization, 200 µL of chloroform were added, and the sample was vortexed until it became turbid, followed by incubation at RT for 15 min. Then, the tube was centrifuged at 12,000 x*g* for 15 min at 4°C to separate the mixture into phases. The aqueous phase, which contained exclusively RNA, was removed and placed into a new tube before proceeding with isolation. For isolation, 500 µL of 100% isopropanol were added to the RNA-containing phase and incubated overnight at -20°C. The samples were then centrifuged at 12,000 x*g* for 10 min at 4°C. The supernatant was removed by inversion, and the RNA pellet was washed twice with 1 mL of 75% ethanol; each wash was followed by centrifugation at 7,400 x*g* for 5 min at 4°C. The supernatant was discarded, and the pellet was air-dried and resuspended in 10-30 µL of RNase-free water (depending on the size of the pellet). Resuspension was optimized by incubation at 55-60°C for 10 min.

Fresh tumor tissues

Before extracting total RNA from tissue, the samples were weighed, defrosted at RT, and cut into small pieces using sterilized scissors. A 1 mL of TRIzol™ reagent was added for each 0.1 g of tissue, and the samples were homogenized with Polytron PT MR3100 (Kinematica AG, Littau, CH) at 11,200 x*g* until the tissue dissolved completely. Then, 200 µL of chloroform were added; the subsequent steps were the same described above for total RNA extraction from peripheral blood.

Formalin-fixed/paraffin-embedded tissues

To increase the yield of RNA recovered from FFPE tumor tissues, we performed the same initial adaptations used for DNA isolation. Each 10-µm tissue slice was deparaffinized, washed with 100% and 70% ethanol and air-dried. Then, 1 mL of TRIzol™ reagent was added. The subsequent steps were the same used to isolate total RNA from fresh blood.

FNA cells in stained slides

To extract RNA from FNA stained slide samples, we resuspended the cells in 1 mL of TRIzol™ reagent by scraping attached cells and pipetting up and down.

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Two washes with 70% ethanol were performed to increase the RNA yield, and to diminish interfering coloration of the precipitated pellet. The subsequent steps were the same used to isolate total RNA from peripheral blood.

Cultured cells

For cells in culture (1-2x10^6 to 1-2x10^7), we first removed the growth media and washed the cells with 1xPBS, as described above, and then added 1 mL of TRIzol™ reagent per 10 cm^2 of culture dish surface area. This cell detaching and lysing step was performed directly in the culture dish by pipetting up and down several times to ensure the complete dissolution of the cells. Next, 200 µL of chloroform were added. The subsequent steps were the same used to isolate total RNA from peripheral blood.

RNA/DNA in-house optimized co-extraction from FNA cells in stained slides and FFPE tumor tissues

Using standard TRIzol™ protocol for co-extraction of RNA/DNA from FNA cells in stained slides and FFPE tumor tissues, DNA from two out of seven samples was not suitable for subsequent use. To improve this recovery, after centrifugation for RNA and DNA separation in two phases, we extracted RNA following the manufacturer's procedures, and precipitated DNA by adding 400 µL of 100% ethanol, incubating the mixture for 3 min at RT, and centrifuging the sample at 2000 x*g* for 20 min at 4°C.The DNA pellet was re-extracted using our in-house method. These steps are represented in figure 3. DNA from FNA cells in stained slides and FFPE tissues presented better quality when compared with standard TRIzol™ protocol (Figure 2).

Polymerase chain reaction

DNA was amplified in 25-µL PCR reactions containing 10 pM of each specific primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM dNTP, 1.5 µM $MgCl_2$, 0.2 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Primers are rigorously designed to avoid SNP regions, using the publicly available primer design method at http://ihg.gsf.de/ihg/ExonPrimer.html. The amplification of DNA isolated from FNA cells in stained slides and FFPE tissues successfully demonstrated the quality of the in-house extraction protocol to recover DNA from these samples (Figure 4).

Bisulfite DNA sequencing for methylation analysis

The bisulfite conversion of genomic DNA was performed using the Methylcode Bisulfite Conversion Kit (Invitrogen). After bisulfite conversion, the *HES1* promoter region was amplified using bisulfite-PCR oligonucleotides that were designed using MethylPrimer Express Software (Applied Biosystems, Foster City, CA, USA). PCR products were cloned into the pCR4 vector (Topo TA Cloning Kit, In-

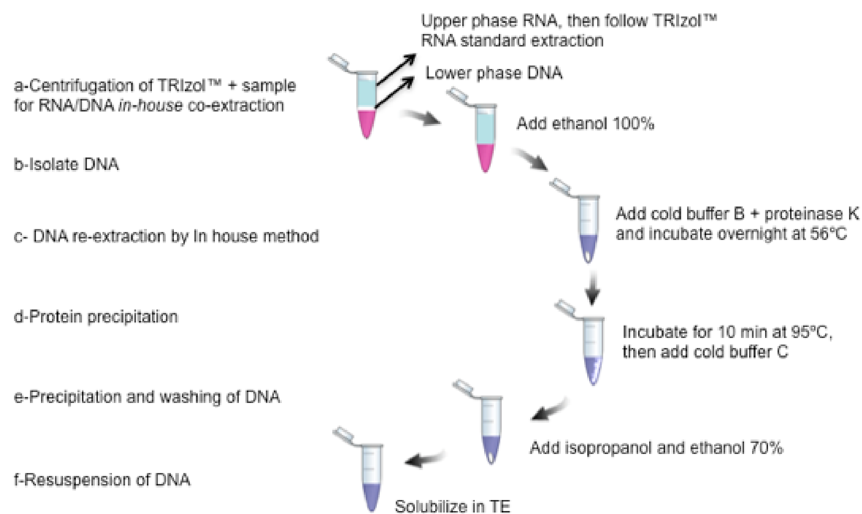

Figure 3. Schematic representation of the major steps for TRIzol™ RNA/DNA in-house optimized co-extraction. RNA and DNA in TRIzol™ are normally separated in an upper and a lower phase, respectively. After that, RNA can be extracted following manufacturer's standard procedures, and DNA can be re-extracted from the lower phase, by means of optimization using our in-house modified salting-out method.

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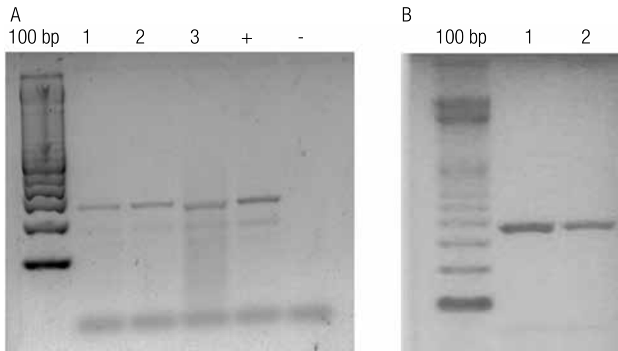

Figure 4. Agarose gel (1.8%) electrophoresis of PCR products. **A:** exon 15 of the *BRAF* gene (245 bp) amplification using DNA samples (1, 2 and 3) of FNA cells in stained slides; (+) positive control, (-) negative control. **B:** amplification of exon 10 of the *RET* gene (374 bp) using DNA samples (1 and 2) from FFPE tissue.

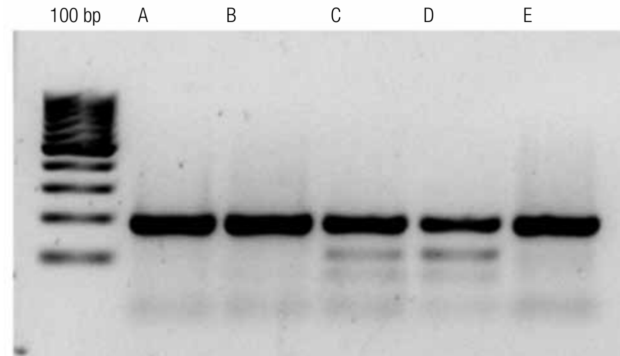

Figure 5. Agarose gel (1.8%) electrophoresis of PCR products representing the promoter region of *HES1* (180 bp). **A-E:** DNA samples from FFPE medullary thyroid cancer treated with sodium bisulfite used as templates. Despite the fragmentation of the DNA in FFPE tissue, amplification after treatment with sodium bisulfite was successful, making epigenetic studies possible.

vitrogen), transformed into DH5'-T1 chemically competent cells, and plated under antibiotic selection. Plasmid DNA from isolated colonies was extracted with QIAPrep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and sequenced to determine *HES1* promoter methylation status. Multiple independent clones were sequenced for each of the amplified fragments.

Direct sequencing of PCR products

PCR products were resolved by electrophoresis in agarose gel, purified using the Illustra GFX PCR DNA and Gel Purification Kit (GE Healthcare, Buckinghamshire, UK), and submitted to direct sequencing by the Sanger method, using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM 3130 *xl* Genetic Analyzer (Applied Biosystems). Each exon was sequenced at least twice and in both directions.

The sequences were analyzed in the BioEdit Sequence Alignment Editor and CLC Main Workbench 6 (http://www.clcbio.com) and compared with reference data available from the NCBI GenBank and the Ensembl Genome Browser. For somatic mutations, we referred to the Catalog of Somatic Mutations in Cancer (COSMIC) database (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Bisulfite sequencing data were analyzed using BiQ Analyzer software v16. To avoid misinterpretation, two different readers analyzed all results.

The HES1 promoter region was amplified from DNA extracted from FFPE medullary thyroid cancer tissues treated with sodium bisulfite, and the resulting PCR products were directly sequenced (Figures 5 and 6).

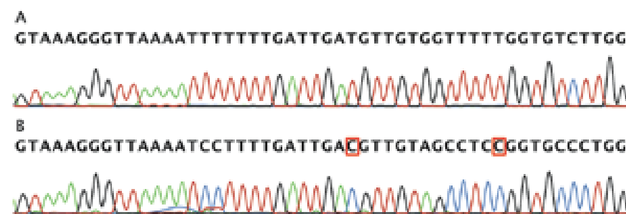

Figure 6. Direct sequencing of the *HES1* promoter region (180 bp) from DNA samples of FFPE medullary thyroid cancer tissue after pretreatment with sodium bisulfite. **A:** whole converted sequence. **B:** highlighted unconverted bases.

Reverse transcription reaction and real-time polymerase chain reaction

Between 0.5 and 1 µg of total RNA recovered from peripheral whole blood, tumor tissue, FFPE tissue, FNA cells in stained slides and cultured cells were used to perform the reverse transcription reaction. DNAse pretreatment was conducted to prevent contamination with genomic DNA. Next, oligodT or random primers, 10 mM of each deoxyribonucleoside phosphate (dNTP), 5x first-strand buffer, 0.1 M dithiothreitol (DTT), RNAse Out Mix (Invitrogen), and 200 U reverse transcriptase (SuperScript II, Invitrogen) were added and reverse transcription was performed at 65°C for 5 min, followed by 1.5 min on ice, 60 min at 50°C, and 15 min at 70°C. Real-time PCR was carried out for the *S8* gene in triplicate in a 20-µL reaction volume of 5-32 ng cDNA, 10 µL SYBR Green Master Mix (Applied Biosystems), and 10 pmol/L of each primer. Fluorescence intensity was quantified, and amplification plots were analyzed by a sequence detector system (ABI Prism 7500; Applied Biosystems), as shown in figure 7.

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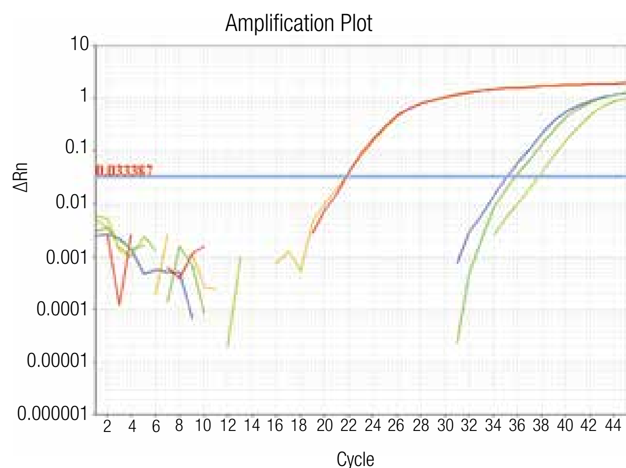

Figure 7. Real-time PCR of the *S8* gene from cDNA from fresh blood (beige line), TT cells (red line), FNA cells in stained slides (blue line), FFPE tissue (dark green), and long-term stored tissue (light green). As expected, FNA cells in stained slides, FFPE and long-term stored tissues presented higher threshold cycles, most likely due to lower RNA quality and integrity. Adaptations to improve the efficiency of these reactions should be pursued.

DISCUSSION

With the growing interest in understanding the genetic basis of many diseases, obtaining enough nucleic acid with reasonable quality for downstream molecular analysis is a constant challenge. In this report, we describe improvements to different procedures carried out in our research laboratory.

In-house protocols for DNA extraction modified from the salting-out technique, have been used for fresh and long-term stored blood samples (6-9). Furthermore, long-term stored blood samples (1-10 years), which are normally discarded in clinical laboratories, can provide valuable material for genetic and epigenetic studies, reducing the number of samples required from the patient, thus saving time and lowering costs (8,10). As proposed by other authors (8,9), we chose heat shock, addition of proteinase K, longer incubations with buffer A, and vortexing as additional strategies to dissolve clots and isolate high quality DNA useful for further analysis.

Analyses of DNA and RNA isolated from FFPE tumor tissue are useful for assessing disease etiology, mechanisms of carcinogenesis, and biomarkers for the prognosis and prediction of treatment responses (1). In this paper, we propose the use of this in-house protocol modified from Goelz and cols. (11) and Bowtell (5) to recover DNA from FFPE tissue samples, which resulted in higher yields of DNA with better quality when compared with commercial kits, possibly due to the higher temperature used in the inactivation of proteinase K and subsequent heat shock, preventing the possible retention of some DNA in the purification column. Additionally, submitting the pellet of precipitated protein (after buffer C addition and centrifugation) to re-extraction, we were able to isolate significant quantities of DNA, in the range of 300 ng/µL. We also noticed that, in some cases, amplification in PCR reactions was better when using DNA extracted with the in-house protocol than when using DNA obtained with commercial DNA extraction kits, that produced amplicons as large as 374 bp, despite the frequent recommendation to design PCR products no larger than 150 to 200 bp (12).

The use of DNA from FFPE tissue for epigenetic analysis can be challenging, as it is known to be highly fragmented, and can be even more degraded after bisulfite treatment, often impairing further methylation profiling (13). Still, with better quality DNA, we have achieved effective amplification and sequencing of bisulfite-treated GC-rich sequences of up to 489 bp in length, despite the recommendation to use fragments smaller than 150 bp in this kind of sample (13).

The major limitations for RNA isolation from FFPE tissue samples are cross-linking between nucleic acids and proteins, and degradation promoted by formalin fixation. In general, pre-treatment with proteinase K and/or digestion buffer prior to RNA isolation procedures is recommended (14-20). In this regard, we have recovered RNA even without using these intermediate steps. These results were similar to those described by other groups using TRIzol™ reagent (19,21), while other groups were unable to achieve successful RNA recovery (22). It seems that the omission of deparaffinization and rehydration steps has improved the efficiency of real-time PCR (data not shown) by reducing sample cross-contamination from repeated pipetting, and by preventing the risk of RNA degradation (23).

For FNA analysis, obtaining material from the same sample that has been analysed by a cytopathologist is important to ensure clinically integrated morphological and molecular diagnosis, especially because of the heterogeneity of thyroid tumors and the difficulty of obtaining identical FNA cytology results from multiple samples (4). DNA purification from FNA cells in stained slides has been reported to present methodological challenges, including low DNA quality and concen-

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tration, especially when using long-term stored slides, and fixation/staining procedures, which result in PCR inhibition (24). To improve current methodologies, we made some modifications to previously described protocols (6,25-27). Our FNA stained slides from thyroid nodules are routinely maintained at RT without coverslips, so pre-treatment with xylene and re-hydration by means of ethanol washes were not necessary. Instead of using cells resuspended in lysis buffer for PCR, as proposed by some authors (28,29), we used purified and precipitated DNA. We believe that not exposing the cells to xylene and the heat shock step result in better DNA quality, improving PCR efficiency. As it is known, the use of the TRIzol™ protocol enables the recovery of both RNA and DNA. Thus, we made an effort to improve the amount of DNA by adding a step of DNA re-extraction, as shown in figure 3. This additional step in DNA extraction from TRIzol™-DNA phase, using buffers B and C, together with the final isopropanol/ethanol precipitation, greatly improved the amount and quality of DNA obtained. As previously mentioned, DNA and RNA extraction from cells in stained slides ensures that the same sample that has been analysed by a cytopathologist is further analysed by molecular methods, which could be extremely relevant when dealing with lesions of uncertain significance.

Regarding the sequencing analysis of these samples, caution and rigor are essential to avoid genotyping errors. The issue that we consider to be most concerning is allelic dropout, i.e., when only one of the two alleles present at a heterozygous locus is amplified (30), which may lead to false negative results. Low quality or quantity of DNA, chemical contaminants, and uneven PCR amplification of sequences with different GC contents can cause this phenomenon. To overcome this amplification bias, we routinely design primers according to recent data on reference sequences to avoid including SNPs in primer binding sites. Other important strategies include optimizing PCR reactions for low-stringency annealing temperatures to prevent the amplification of unspecific bands, together with the use of a high-fidelity, high-specificity and GC-rich performance proof-reading DNA polymerase. Additionally, sequence analysis should be performed cautiously when evaluating SNP in heterozygosis; the absence of SNPs in an exon known to have many polymorphisms, or the presence of a homozygous SNP might suggest preferential PCR amplification. To minimize possible misreading, we included at least two independent electropherogram sequence readers, using different genome software analyzers. In addition, a second PCR/sequencing reaction was performed to check the results.

CONCLUSION

In general, we propose the use of proteinase K and heat shock for fresh and long-term stored blood, FFPE tissue, and FNA cells in stained slides for greater DNA yield. For RNA extraction from blood samples, we suggest measures to prevent clot formation. In addition, by making adaptations to the in-house and standard TRIzol™ protocols, and optimizing co-extraction of RNA and DNA from the same specimen, we successfully isolated these nucleic acids sequentially from FFPE tissue and from long-term stored cells in stained slides (from fine-needle aspiration), which are known as scarce sources of nucleic acids of great importance for molecular diagnosis in endocrinology. DNA and RNA isolated from all these samples were suitable for PCR, methylation profiling, real-time PCR, and DNA sequencing analyses, enabling subsequent molecular diagnosis.

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REFERENCES

1. Huang WY, Sheehy TM, Moore LE, Hsing AW, Purdue MP. Simultaneous recovery of DNA and RNA from formalin-fixed paraffin-embedded tissue and application in epidemiologic studies. Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2010;19(4):973-7.
2. Pikor LA, Enfield KS, Cameron H, Lam WL. DNA extraction from paraffin embedded material for genetic and epigenetic analyses. JoVE. 2011(49).
3. Kotoula V, Charalambous E, Biesmans B, Malousi A, Vrettou E, Fountzilas G, et al. Targeted KRAS mutation assessment on patient tumor histologic material in real time diagnostics. PloS One. 2009;4(11):e7746.
4. Ferraz C, Eszlinger M, Paschke R. Current state and future perspective of molecular diagnosis of fine-needle aspiration biopsy of thyroid nodules. J Clin Endocrinol Metab. 2011;96(7):2016-26.

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5. Bowtell DD. Rapid isolation of eukaryotic DNA. Anal Biochem. 1987;162(2):463-5.
6. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988;16(3):1215.
7. Buffone GJ, Darlington GJ. Isolation of DNA from biological specimens without extraction with phenol. Clin Chem. 1985;31(1):164-5.
8. Adkins KK, Strom DA, Jacobson TE, Seemann CR, O'Brien DP, Heath EM. Utilizing genomic DNA purified from clotted blood samples for single nucleotide polymorphism genotyping. Arch Pathol Lab Med. 2002;126(3):266-70.
9. Di Pietro F, Ortenzi F, Tilio M, Concetti F, Napolioni V. Genomic DNA extraction from whole blood stored from 15- to 30-years at -20 degrees C by rapid phenol-chloroform protocol: a useful tool for genetic epidemiology studies. Mol Cell Probes. 2011;25(1):44-8.
10. Crider KS, Quinlivan EP, Berry RJ, Hao L, Li Z, Maneval D, et al. Genomic DNA methylation changes in response to folic acid supplementation in a population-based intervention study among women of reproductive age. PloS One. 2011;6(12):e28144.
11. Goelz SE, Hamilton SR, Vogelstein B. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochem Biophys Res Commun. 1985;130(1):118-26.
12. Bonin S, Petrera F, Niccolini B, Stanta G. PCR analysis in archival postmortem tissues. Mol Pathol. 2003;56(3):184-6.
13. Patterson K, Molloy L, Qu W, Clark S. DNA methylation: bisulphite modification and analysis. J Vis Exp. 2011(56). pii: 3170.
14. Godfrey TE, Kim SH, Chavira M, Ruff DW, Warren RS, Gray JW, et al. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5' nuclease quantitative reverse transcription-polymerase chain reaction. J Mol Diagn. 2000;2(2):84-91.
15. Krafft AE, Duncan BW, Bijwaard KE, Taubenberger JK, Lichy JH. Optimization of the Isolation and Amplification of RNA From Formalin-fixed, Paraffin-embedded Tissue: The Armed Forces Institute of Pathology Experience and Literature Review. Mol Diagn. 1997;2(3):217-30.
16. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res. 1999;27(22):4436-43.
17. Jackson DP, Lewis FA, Taylor GR, Boylston AW, Quirke P. Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. J Clin Pathol. 1990;43(6):499-504.
18. Dedhia P, Tarale S, Dhongde G, Khadapkar R, Das B. Evaluation of DNA extraction methods and real time PCR optimization on formalin-fixed paraffin-embedded tissues. Asian Pac J Cancer Prev. 2007;8(1):55-9.
19. Korbler T, Grskovic M, Dominis M, Antica M. A simple method for RNA isolation from formalin-fixed and paraffin-embedded lymphatic tissues. Exp Mol Pathol. 2003;74(3):336-40.
20. Koopmans M, Monroe SS, Coffield LM, Zaki SR. Optimization of extraction and PCR amplification of RNA extracts from paraffin-embedded tissue in different fixatives. J Virol Methods. 1993;43(2):189-204.
21. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162(1):156-9.
22. Witchell J, Varshney D, Gajjar T, Wangoo A, Goyal M. RNA isolation and quantitative PCR from HOPE- and formalin-fixed bovine lymph node tissues. Pathol Res Pract. 2008;204(2):105-11.
23. Lehmann U, Kreipe H. Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. Methods. 2001;25(4):409-18.
24. Canfell K, Gray W, Snijders P, Murray C, Tipper S, Drinkwater K, et al. Factors predicting successful DNA recovery from archival cervical smear samples. Cytopathology. 2004;15(5):276-82.
25. Mattu R, Sorbara L, Filie AC, Little R, Wilson W, Raffeld M, et al. Utilization of polymerase chain reaction on archival cytologic material: a comparison with fresh material with special emphasis on cerebrospinal fluids. Mod Pathol. 2004;17(10):1295-301.
26. Marchetti I, Lessi F, Mazzanti CM, Bertacca G, Elisei R, Coscio GD, et al. A morpho-molecular diagnosis of papillary thyroid carcinoma: BRAF V600E detection as an important tool in preoperative evaluation of fine-needle aspirates. Thyroid. 2009;19(8):837-42.
27. Poljak M, Barlic J, Seme K, Avsic-Zupanc T, Zore A. Isolation of DNA from archival Papanicolaou stained cytological smears using a simple salting-out procedure. Clin Mol Pathol. 1995;48(1):M55-6.
28. Smith GD, Chadwick BE, Willmore-Payne C, Bentz JS. Detection of epidermal growth factor receptor gene mutations in cytology specimens from patients with non-small cell lung cancer utilising high-resolution melting amplicon analysis. J Clin Pathol. 2008;61(4):487-93.
29. de Roda Husman AM, Snijders PJ, Stel HV, van den Brule AJ, Meijer CJ, Walboomers JM. Processing of long-stored archival cervical smears for human papillomavirus detection by the polymerase chain reaction. Br J Cancer. 1995;72(2):412-7.
30. Pompanon F, Bonin A, Bellemain E, Taberlet P. Genotyping errors: causes, consequences and solutions. Nat Rev Genet. 2005;6(11):847-59.

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