M. Jarząb[1], P. Różanowski[2], M. Kowalska[1], J. Żebracka[1], L. Rudnicka[3], E. Stobiecka[1], B. Jarząb[1], J. Stachura[3], J. Pawlęga[2]

Optimization of the Method of RNA Isolation from Paraffin Blocks to Assess Gene Expression in Breast Cancer

[1]Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Gliwice,
[2]Chair and Department of Oncology, Collegium Medicum, Jagiellonian University, Kraków,
[3]Chair of Clinical and Experimental Pathomorphology, Collegium Medicum, Jagiellonian University, Kraków

Molecular oncology increasingly needs the assessment of tumor gene expression profile (transcriptome), most commonly by determination of RNA-based molecular markers employing the technique of quantitative real-time polymerase chain reaction (Q-PCR). However, as all are methods based on RNA, to date, the experience in Q-PCR is mostly limited to freshly collected material frozen at -80^{0}C, i.e. showing no signs of RNA degradation. The aim of the present study was to implement into practice a method of RNA isolation from formalin-fixed and paraffin-embedded (FFPE) breast carcinoma samples collected during routine surgical and histopathological procedure, to further employ it in expression analysis by Q-PCR.

The RNA isolation kit RNeasy FFPE (QIAGEN) was used. It was demonstrated that in samples subjected to DNAse digestion, the mean concentration of the obtained RNA was low (46 ng/µl), while during the isolation performed using solely gDNA Eliminator columns, the authors obtained RNA with an almost fourfold higher concentration value. A comparison was made between isolation effectiveness using varying amounts of input material. It was noted that isolation efficacy was lower when three sections were employed (the concentration value of 178 ng/µl) as compared to 5-8 sections (279 and 302 ng/µl, respectively). RNA quality assessment was also performed employing the method of capillary electrophoresis by the „lab-on-a-chip" technology of Agilent Bioanalyzer 2100. Freshly prepared material yielded in single cases samples containing RNA18S and RNA28S populations, while in samples isolated from archival paraffin blocks, the obtained RNA showed more considerable degradation, thus, was of lesser quality. In the analysis of 20 samples from the second collected series, the majority of samples were characterized by the RNA Integrity Number (RIN) values in the range of 2-2.5, still indicative of a substantial degree of RNA degradation. The mean isolation effectiveness in the second series was 885 ng/µl. In 10 of 20 blocks isolated, we succeeded in obtaining sufficient RNA concentration, above 500 ng/µl. It was also noted that the storage time did not affect the amount of RNA obtained from a block: while isolating RNA from freshly prepared blocks, we achieved similar concentrations as when analyzing the archival material.

Conclusions: the key in preserving RNA quality in paraffin blocks is the timing of material collection and fixing. Routine paraffin blocks allow for obtaining RNA for molecular studies, yet with features of considerable degradation.

Introduction

The analysis of a wide spectrum of prognostic and predictive factors seems to be the crucial aspect of individualized therapy in oncology. At the same time, the increasing number of tailored molecular therapies compels the clinicians to appropriately identify patients who potentially qualify for therapy directed at precisely defined cellular mechanisms [1, 17]. To date, a routine analytical method for molecular markers assessment in oncology is immunohistochemistry (IHC), based on protein analysis employing specific antibodies. For assessment of single DNA markers, the method of fluorescent in situ hybridization (FISH) has been developed; the method is based on assessment

of genomic DNA using a specific oligonucleotide probe. However, the two above-mentioned methods (IHC, FISH) have significant limitations, chiefly resulting from the fact that they are labor-consuming and the results are difficult to objectivize. Moreover, both methods are perfectly suitable for investigating expression of single markers; but their use in the case of a wide spectrum of genes/proteins markedly increases the consumption of biological material and prolongs the time of examination. In the context of an increasing need in oncology of performing evaluation of the entire profile of gene expression in a tumor, a more and more commonly proposed alternative consists in determination of molecular markers based on RNA, for example employing the technique of quantitative real-time polymerase chain reaction (Q-PCR) [13]. The method, based on amplification of gene transcripts, allows for analyzing a large number of markers in a small amount of material; at the same time, it may be easily adapted to investigations of new, hitherto unknown genes. Nevertheless, as in the case of all RNA-based methods, to date, the experience in employing Q-PCR is mostly limited to freshly collected material frozen at -80 ^{0}C, i.e. showing no signs of RNA degradation.

The objective of the present paper is a preliminary introduction into practice of a method of RNA isolation from formalin-fixed and paraffin-embedded (FFPE) breast carcinoma samples collected during routine histopathologic diagnostic management, with a perspective of their future employment in expression analysis by Q-PCR.

Material and Methods

The material for the present biomolecular investigations originated from paraffin blocks stored at the archives of the Chair of Clinical and Experimental Pathomorphology, Collegium Medicum, Jagiellonian University, Kraków (Head: Professor Jerzy Stachura, MD, Ph.D.). The blocks represented material from females with primary operative breast carcinomas subjected to radical mastectomies in surgical departments of the University Hospital of Krakow in the years 1994-2003, and subsequently to adjuvant therapy and/or follow-up in the Chair and Department of Oncology, Collegium Medicum, Jagiellonian University, Kraków (Head: Professor Janusz Pawlęga, MD, Ph.D.). Studies on gene expression were performed at the Laboratory of Functional Genomics and Molecular Diagnostics, Department of Nuclear Medicine and Endocrine Oncology, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch (Head: Professor Barbara Jarząb, MD, Ph.D.).

RNA isolation was performed using the QIAGEN RNeasy FFPE Kit (Qiagen, Hilden, Germany), according to the protocol developed by the manufacturer. The RNeasy FFPE kit includes traditional RNeasy MinElute series columns for RNA purification and additionally gDNA Eliminator columns, which allow for removing from the sample genomic DNA that contaminates the isolated RNA.

In the analysis, two series of paraffin blocks were employed (16 blocks in the first, and 20 – in the second analysis). Additionally, five blocks were prepared from fresh postoperative material; the blocks were processed in the standard way and stored for one week prior to RNA isolation procedure. The blocks were microtomed into 6-7 μm-thick sections. In RNA isolation, 3-8 sections were used per one examination. The material was deparaffinized using various volumes of xylene (from 0.5 to 2 ml), and subsequently, following supernatant removal, the sample was rinsed in alcohol.

RNA concentration was evaluated using the Nanodrop ND-1000 microspectrophotometer, while RNA quality was assessed employing the Agilent 2100 Bioanalyzer, RNA 6000 Nano kits and the RIN algorithm (RNA Integrity Number).

Results

In the first stage of the investigation, the authors assessed the validity of the classic isolation technique developed by Chomczynski-Sacchi, where RNA is purified using the QIAGEN RNeasy Mini columns and DNAse-digested. This is a routine procedure for RNA isolation from frozen sections and RNAlater-fixed samples. In view of the fact that the results obtained with the above procedure were highly unsatisfactory and the resultant RNA had the concentration of approximately 10 ng/μl, we attempted RNA isolation using the QIAGEN RNeasy FFPE kit, which includes proteinase K-digestion, elimination of genomic DNA contamination in microcolumns and RNA isolation using RNeasy columns.

As the first step of the analysis, we checked the effect of DNAse digestion of a sample on isolation efficiency. It was demonstrated that in samples subjected to DNAse digestion (and subsequently isolated using RNeasy and gDNA Eliminator columns), the mean concentration of the obtained RNA was low (46 ng/μl), while isolation performed employing solely the gDNA Eliminator columns yielded RNA with an almost four times higher concentration value (mean, 178 ng/μl, Fig. 1).

Subsequently, we compared the efficiency of isolation employing varying amounts of input material (3, 5 or 8 sec-

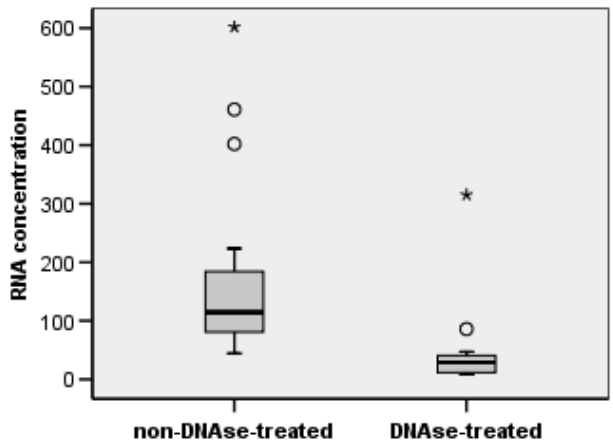

Fig. 1. Comparison of RNA concentration values obtained following isolation with and without the DNAse digestion step in the column.

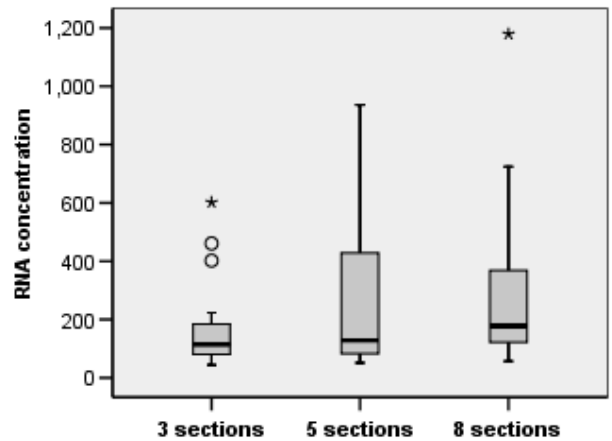

Fig. 2. Comparison of RNA isolation effectiveness at varying amounts of input material (number of sections).

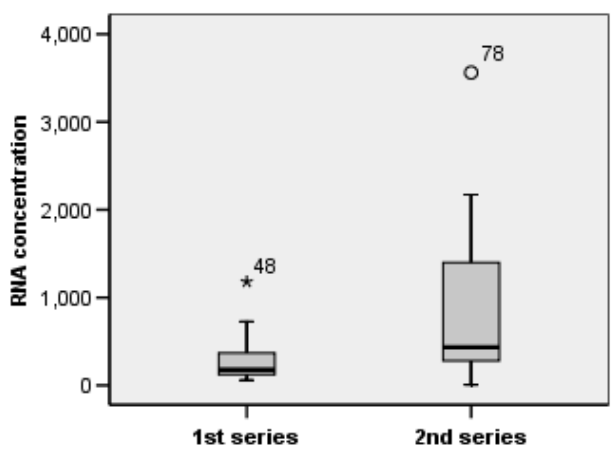

Fig. 3. Comparison of results obtained in the first and second isolation series.

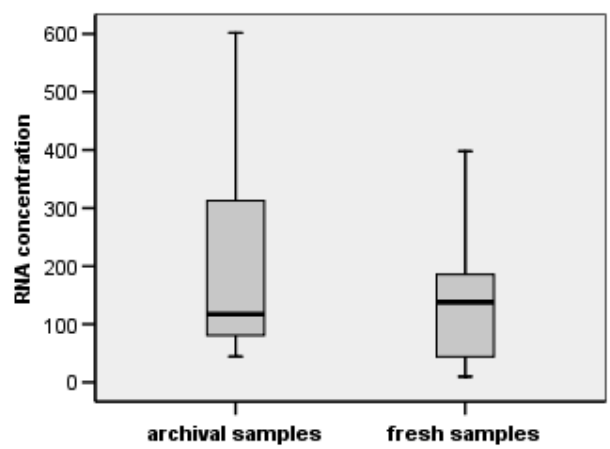

Fig. 4. Comparison of results of RNA isolation from archival and freshly prepared materials.

tions per isolation). It was noted that isolation efficiency was lower when three sections were employed (the concentration value of 178 ng/µl) as compared to 5-8 sections (279 and 302 ng/µl, respectively), while the increase of efficiency associated with increasing the number of sections from five to eight was less pronounced (Fig. 2). In addition, while evaluating the usefulness of an additional step consisting in double deparaffinization in xylene, we did not find the procedure to contribute to a consistent increase in isolation effectiveness.

A clear improvement in the amount of the obtained RNA was observed, being most likely associated with the learning curve. The mean isolation effectiveness, which was 302 ng/µl in the first series of analyses (isolation from eight sections), in the second series increased up to 885 ng/µl (Fig. 3). In 10 of 20 blocks isolated in the second series, we succeeded in obtaining RNA concentration above 500 ng/µl, and thus the increased total amount of RNA to several micrograms, what was a satisfactory result, allowing for further analyses. It was also noted that the time of storage did not affect the amount of RNA obtained from a block: while isolating RNA from freshly prepared blocks, similar concentration values were obtained as when analyzing archival materials (Fig. 4).

Quality assessment was also performed in RNA obtained in the above-mentioned manner employing the method of capillary electrophoresis and the „lab-on-a-chip" technology of Agilent Bioanalyzer 2100. While analyzing groups of blocks originating from archival materials as well as freshly prepared blocks, we observed significant differ-

ences in the quality of isolated RNA. Although using freshly prepared materials, in single cases we managed to obtain samples containing RNA 18S and 28S populations (Fig. 5A and B, Fig. 6), in samples originating from the archives, the material showed considerable degradation and no striae lines corresponding to RNA 18S and 28S were visible (Fig. 5C). The fact that in all the examined archival samples no ribosomal striae lines of RNA 18S and 28S have been obtained is in agreement with isolation results achieved by other laboratories employing paraffin blocks.

In the analysis of 20 samples from the second series, the majority of samples were characterized by the RIN values in the range of 2-2.5, indicative of a high degree of RNA degradation. Only a single sample did demonstrate RIN approximating the minimum value (1), characteristic for extreme RNA degradation (Fig. 7).

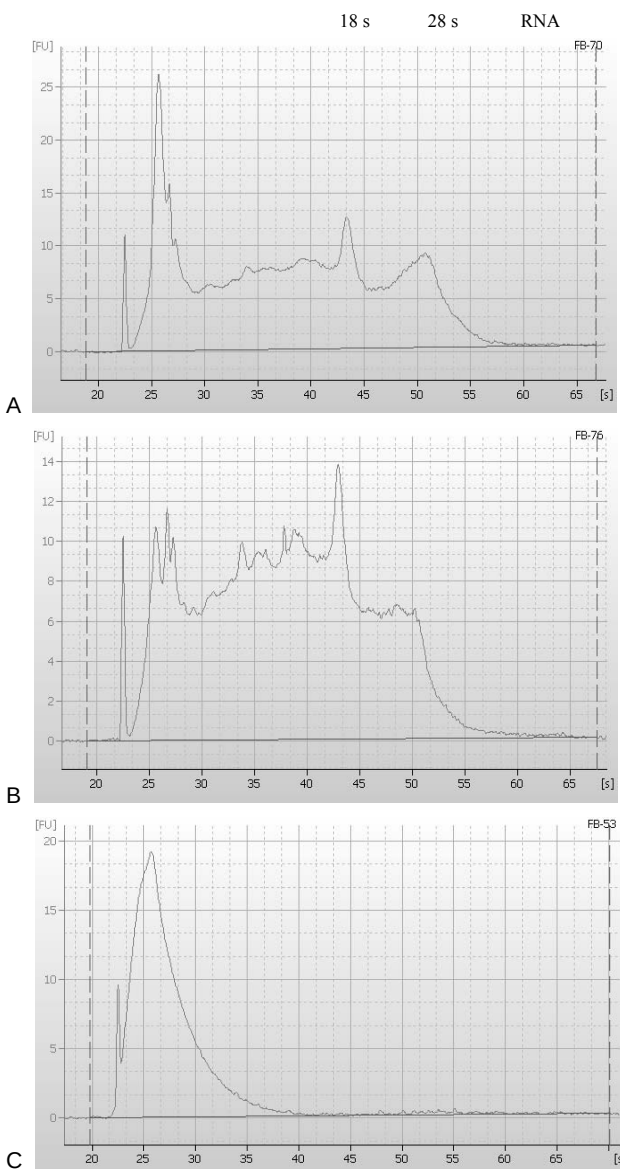

Fig. 5. Results of qualitative RNA assessment by capillary electrophoresis. In the case of samples isolated from freshly prepared paraffin blocks (A, B), the electrophoregram shows 18S and 28S ribosomal RNA fractions (arrows). In the case of the majority of samples isolated from archival material, these fractions are not visible; an example of the result is presented in Fig. C.

Discussion

At present, research efforts are made to determine a set of molecular markers with prognostic and/or predictive importance in breast cancer. Such studies are supposed to

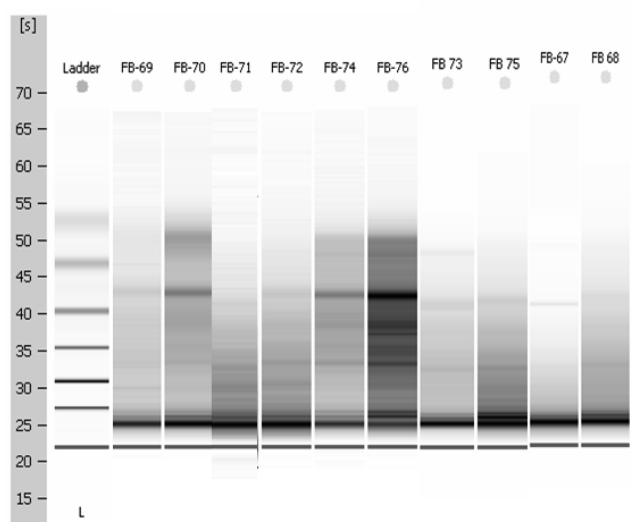

Fig. 6. Results of capillary electrophoresis exemplified by ten samples isolated from paraffin blocks.

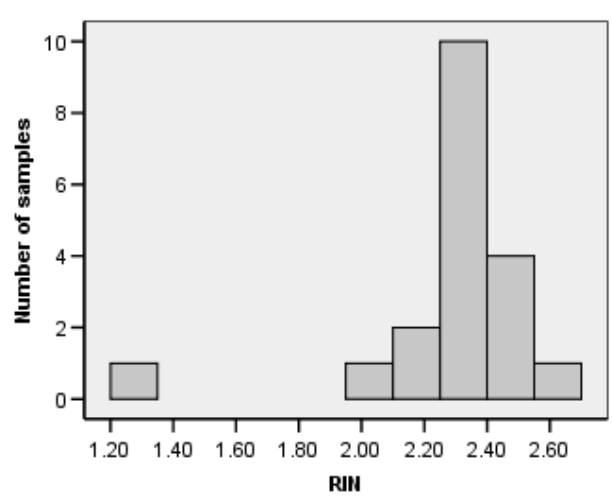

Fig. 7. A histogram of the RIN index in the examined samples.

TABLE 1
Examples of isolation results from archival material (sample concentration values given in ng/μl)

Block No.	3 sections, additional DNAse-digestion	3 sections	3 sections, double rinsing in xylene	5 sections	8 sections
133726	38	461	556	435	549
1288697	86	402	367	732	725
1282259	36	117	132	174	116
132985	22	71	31	118	183
1331039	44	112	126	126	156
1328826	8	77	25	64	57

TABLE 2
Examples of isolation results from archival material (the RIN index values are given)

Block No.	3 sections, additional DNAse-digestion	3 sections	3 sections, double rinsing in xylene	5 sections	8 sections
133726	2.3	2.3	2.3	2.4	2.4
1288697	2.4	2.2	1.9	2	2.1
1282259	2.3	2.4	2.5	2.4	2.5
132985	2.4	2.5	---	2.5	2.3
1331039	2.5	2.5	2.4	2.5	2.5
1328826	2.5	2.5	2.2	2.5	2.5

specify novel molecular markers in breast carcinoma, associated with the best reaction (or lack of such a reaction) to a particular type of treatment or providing a reliable source of information on the prognosis [22, 24, 25, 26].

To date, molecular studies in oncology have been generally based on analysis of alterations in the structure/expression of single genes or small gene groups. Nevertheless, so far, information yielded by single markers (e.g. ER/PgR, HER-2, TOPO2A, BRCA-1/2) does not allow for obtaining reliable predictors of survival or response to treatment. Hence, rather than "classic" methods (IHC), it is proposed to employ methods based on DNA or RNA analysis. Unquestionable disadvantages of IHC include: 1) dependence of results on numerous additional factors, such as the epitope type, method of antibody detection, method of tissue fixing (freezing, paraffin-embedding), conditions and time of postoperative material storing and processing (fixative, staining, time interval between surgical resection and fixation), 2) subjectivity of result evaluation, and 3) lack of uniform criteria of assessing the level (threshold) of "positive" and "negative" results (the threshold intensity of color enzymatic reaction).

The basic DNA-based method of molecular pathology is the FISH method. The method has significant limitations: 1) a high cost, 2) a higher labor expenditure as compared to IHC, 3) a necessity to employ a fluorescence microscope. To provide a reliable evaluation, both methods require a relatively large amount of tissue material to be studied, and at the same time, the very assessment, especially in the case of IHC, is often of an arbitrary character.

In view of the limitations of the techniques employed to date, investigators consider using such methods as DNA microarrays or quantitative real time PCR. Breast cancer is the first tumor where the results of studies on expression profile by the method of microarray have been directly translated into an attempt at employing such results in clinical decision-making processes and the method itself is tested in a prospective, randomized multicenter trial [24, 25]. A large of information obtained from multi-gene classifiers favors the extensive testing of the method in clinical practice [4, 22, 26], with emphasis on assessment of both the prognosis and the reaction to treatment [3,13].

Unfortunately, investigations employing the microarray method require material that would be intraoperatively fixed by deep freezing in –80 ^{0}C. For numerous patients and many centers such a change in the hitherto employed method of postoperative material processing seems not feasible, hence intensive testing of methods al-

lowing for examining material that has been fixed as paraffin blocks [7]. RNA isolation from material previously treated with formaldehyde differs in requirements from classic techniques employed while processing frozen material, routinely used by analytical techniques of gene expression [5, 12]. This is predominantly due to a considerable degradation of the material, formaldehyde-evoked RNA modifications (which are not detectable by classic methods of RNA quality control, such as gel or capillary electrophoresis), as well as organic contaminants originating from the fixation procedure.

Pioneer investigations of Paik et al. [20, 21] demonstrated that examinations of stored paraffin-embedded postoperative breast cancer material is possible; moreover, the investigators have confirmed the clinical validity of determining the expression of 21 selected genes, which formed the so-called recurrence score (RS). It has a prognostic and predictive value, although it is seen mainly in females with early-stage pN0 and ER(+) breast cancer.

There are at least preliminary indications that the prognostic and predictive value of the 21-gene „recurrence score" most likely surpasses hitherto known markers and systems of breast cancer patients classification; these patients are often arbitrarily ascribed to a given risk group. The positive results of studies employing the group of genes selected by Paik have made a great contribution to the development of the OncotypeDX test, which has been accepted by FDA [10] and experts representing ASCO [16] and has been introduced into clinical practice in the United States.

To date, numerous investigations have been performed on the methodology of RNA isolation and gene expression determination by QPCR [2, 11, 18, 23]. Various groups of authors, mostly from the United States, assessed the methodology in studies on gene expression [8, 9, 14, 15]; also in recent period [6, 19] – the method seems to be a valuable and a relatively resistant to technical problems research tool. Nevertheless, the success of a procedure markedly depends on the quality of material employed in molecular studies, and thus indirectly on the quality of material collection and fixation procedures. Hence, we have undertaken a study of the method based on paraffin-embedded samples while employed in routine surgical material. It has been demonstrated that the procedure of fixing and storing significantly increases RNA degradation, but it is still possible to obtain a pool of nucleic acids for analyses. On the other hand, samples collected and processed through fixing employing the „fast-track" procedure have shown RNA of a better quality as compared to routine samples.

The presently initiated method of RNA isolation from paraffin blocks will be a basis for commencing the analysis of the panel of marker genes in the investigated material, thus providing a potential opportunity for obtaining valuable prognostic information in patients with breast cancer.

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Address for correspondence and reprint requests to:
Dr Paweł Różanowski
Chair and Department of Oncology Collegium Medium,
Jagiellonian University
Ul. Śniadeckich 10
31-031 Kraków
Email: klonk@cm-uj.krakow.pl