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Original article

From RNA isolation to microarray analysis: Comparison of methods in FFPE tissues

Nevin Belder[a], Öznur Coskun[b], Beyza Doganay Erdogan[c], Ozlem Ilk[d], Berna Savas[b], Arzu Ensari[b], Hilal Özdağ[a,*]

[a] Ankara University, Biotechnology Institute, Ankara, Turkey
[b] Ankara University, School of Medicine, Department of Pathology, Ankara, Turkey
[c] Ankara University, School of Medicine, Department of Biostatistics, Ankara, Turkey
[d] Middle East Technical University, Department of Statistics, Ankara, Turkey

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ABSTRACT

Background: Genome-wide gene expression profiling analysis of FFPE tissue samples is indispensable for cancer research and provides the opportunity to evaluate links between molecular and clinical information, however, working with FFPE samples is challenging due to extensive cross-linking, fragmentation and limited quantities of nucleic acid. Thus, processing of FFPE tissue samples from RNA extraction to microarray analysis still needs optimization.
Materials and methods: In this study, a modified deparaffinization protocol was conducted prior to RNA isolation. Trizol, Qiagen RNeasy FFPE and Arcturus PicoPure RNA Isolation kits were used in parallel to compare their impact on RNA isolation. We also evaluated the effect of two different cRNA/cDNA preparation and labeling protocols with two different array platforms (Affymetrix Human Genome U133 Plus 2.0 and U133_X3P) on the percentage of present calls.
Results: Our optimization study shows that the Qiagen RNeasy FFPE kit with modified deparaffinization step gives better results (RNA quantity and quality) than the other two isolation kits. The Ribo-SPIA protocol gave a significantly higher percentage of present calls than the 3′ IVT cDNA amplification and labeling system. However, no significant differences were found between the two array platforms.
Conclusion: Our study paves the way for future high-throughput transcriptional analysis by optimizing FFPE tissue sample processing from RNA isolation to microarray analysis.

1. Introduction

Gene expression analysis using microarray is one of the most commonly used tools in cancer studies. Novel molecular tumor subtypes and target prognostic genes have been determined via genome-wide expression profiling using microarray analysis. The clinical utility of knowledge obtained from microarray data has been studied for several tumor types [1]. For example one gene, Zap-70, was defined in chronic lymphocytic leukemia (CLL) disease for prognostic marker using microarray analysis. Also two molecularly distinct forms of Diffuse Large B-Cell Lymphoma (DLBCL) were identified with genome-wide gene expression profile analysis [2,3]. Oncotype DX® Colon Cancer Assay (Genomic Health, Inc., Redwood City, CA) and ColoPrint® (Agendia, BV, Amsterdam, Holland) are another important output of microarray-based gene expression profiling studies. These two tests which were derived from microarray gene expression profile have been validated and both of them are used for prediction of benefit from adjuvant therapy [4].

Sample type and quality are among the major factors affecting the performance of microarray studies. In most such studies, high-quality RNA from fresh-frozen (FF) tissue is used [5]. However, FF tumor samples are not widely available because most tumor samples are routinely fixed in formalin and embedded in paraffin blocks in hospitals [6]. FF tumor sample collection and storage provide another challenge due to various logistical problems [7]. In cancer research, the use of formalin-fixed, paraffin-embedded (FFPE) tissue samples for microarray studies is necessary and almost an obligation [7–10]. FFPE is the standard and most widely available tissue preservation method, with FFPE samples providing comprehensive information for patient diagnosis and prognosis [11,12].

* Corresponding author at: Ankara University, Biotechnology Institute, 06100 Tandogan, Ankara, Turkey.
E-mail address: hilalozdag@gmail.com (H. Özdağ).

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However, FFPE preparation process and storage have distinctly negative impacts on both DNA and RNA quality [13]. More specifically, the most common molecular changes in FFPE tissues caused by formalin are the formation of cross-links between proteins and nucleic acids, along with nucleic acid degradation. These molecular changes render FFPE-isolated nucleic acids incompatible for downstream high-throughput molecular techniques, such as microarray gene expression profiling [12,14]. Nevertheless, several studies have reported that gene expression profiling data can be obtained from FFPE samples [14–16], and efforts to optimize the processing of FFPE tissue samples from nucleic acid extraction to microarray analysis are still continuing.

In response to the growing need to use FFPE samples in molecular studies, various platforms and techniques have been developed. The GeneChip® Human X3P Array was designed specifically for genome-wide gene expression profiling of FFPE samples by Affymetrix. It is based on the Human Genome U133 plus 2.0 Array. The target sequences on the X3P Array are identical to HG U133 Plus 2.0 Array, for a total of 47,000 transcripts with 61,000 probe sets. But, these two types of arrays are significantly different in terms of the probes for X3P array. When compared with the standard GeneChip arrays, X3P arrays are also designed to focus on interrogating sequences located closer to the 3′ end of the transcripts. But in contrast to standard GeneChip arrays, the probe selection criteria have been modified for X3P arrays due to characteristics of FFPE samples. The probes are selected from the last 300 bases, the most 3′ end of the transcripts. Whereas for the standard Affymetrix GeneChip Arrays probes are selected within the region 600 bases proximal to the 3′ ends.

One of the most important approaches is RiboSPIA (Nugen Ovation™ FFPE WTA System, CA, USA), which uses a linear, isothermal, whole-transcriptome amplification system from total RNA for cDNA amplification of RNA extracted from FFPE samples. In this system, the RNA template is amplified using both oligo dT and random primers [17,18]. Priming RNA with random hexamer is advantageous for FFPE samples because adenine residues are more affected than the other bases during formalin incubation. It was previously reported that using random hexamer in cDNA synthesis reaction gave more successful results from RNA-extracted FFPE than oligo dT primer [18–20].

The choice of workflow in microarray studies using FFPE tissue materials is very important. In this respect, several studies have analyzed the effect of different RNA extraction methods, different cDNA synthesis, amplification kits and array platforms on the results obtained from FFPE tissue extracted RNA samples. Comparison of the gene expression profiling results of FFPE samples with matched frozen tissue samples showed that FFPE samples can be used for gene expression analysis [7,14–16,21]. However, despite these important results, none of these studies has presented a clear and robust workflow for FFPE studies from deparaffinization to microarray analysis. Thus, further studies are needed to optimize and standardize the complete workflow protocol. Doing so will prepare the ground for applying high-throughput transcriptional analysis to retrospective prognostic studies in human cancers.

In this study, we propose a robust protocol from deparaffinization to microarray hybridization. To do this, we evaluated the effect of a deparaffinization protocol on RNA yield and purity for the Qiagen RNeasy FFPE kit (Fig. 2a). We then performed a comparative analysis with different RNA extraction kits, cDNA synthesis and labeling protocols, and two microarray platforms from total RNA extracted from microdissected tumor cells of FFPE samples (Fig. 2b). We determined which of the three different commercial kits offered the best RNA extraction method. After identifying this method, we evaluated the effects of two different (Affymetrix 3′ IVT kit and Nugen Ovation FFPE WTA System + Encore Biotin Module) cDNA preparation and labeling protocols on the percentage of present calls. Samples were hybridized to Affymetrix Human Genome U133 Plus 2.0 and U133_X3P microarrays for performance comparison. This study shows that, by careful choice of workflow method, it is possible to obtain consistent and reliable microarray data for genome-wide expression profiling from FFPE materials.

2. Materials and methods

2.1. FFPE tissue samples

FFPE colon cancer tissue sections from four patients, obtained from Ankara University, Faculty of Medicine, Department of Pathology, were used for this study, with ethical approval being obtained from the local ethics committee (Research Ethics Committee of Medical Faculty of Ankara University, Ankara Ref: 153-4854). FFPE tumor samples were between 6 and 10 years old (collected from 2005 to 2009). The FFPE tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. For our study, 4 sections of 8 μm thickness were cut from each sample and placed on clean standard glass slides. To eliminate cross-contamination among the samples, a new sterile microtome blade was used for each parafin block. The work place, microtome and water bath were cleaned with RNase-Zap (Sigma, St. Louis, MO, USA). One tissue section from each sample was stained with hematoxylin and eosin (H&E) to identify the border around the target tumor cells areas for macrodissection. Tumor cells were evaluated by pathologists under a microscope.

2.2. Deparaffinization and determination of optimal RNA isolation techniques

All working areas, instrument surfaces and pipets were treated with RNaseZap solution. RNase-free tips and microtubes were used throughout the study. There is no specified deparaffinization protocol for Trizol and Arcturus PicoPure RNA Isolation kits. However, Qiagen RNeasy FFPE kit has its own deparaffinization protocol. In this study a deparaffinization protocol depicted in Fig. 1a is used for Trizol and PicoPure RNA Isolation kit. This protocol and Qiagen's own deparaffinization protocol (Fig. 1b) are used prior to Qiagen RNeasy FFPE kit comparatively.

Four FFPE tissue sections of 8 μm thickness were deparaffinized as stated in Fig. 1a. This deparaffinization protocol was compared with the Qiagen RNeasy FFPE kit deparaffinization step (Fig. 1b). This modified protocol was then used for all further RNA isolation methods. After deparaffinization, the tumor area was examined under a microscope by a pathologist to determine that the tumor area contained ~90% tumor cells. Tumor-containing regions were scraped in a nuclease-free microcentrifuge tube with a sterile lancet while examining the cells under the microscope.

Total RNA was isolated from the four FFPE tissue sections of 8 μm thickness (6–10-years-old) using three commercially available kits: RNeasyFFPE kit (Qiagen, Hilden, Germany), PicoPureRNA Isolation kit (Arcturus, Mountainview, CA) and TriReagent (Sigma, St. Louis, MO, USA). All extraction protocols were performed according to the manufacturers' instructions with the same deparaffinization (modified) protocol depicted in Fig. 1a.

The RNA concentration and purity (OD, A260/A280 and A260/A230) of the total RNA sample were determined by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was evaluated using the RNA6000 Nano Assay on Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).