Minireview

Simultaneous Recovery of DNA and RNA from Formalin-Fixed Paraffin-Embedded Tissue and Application in Epidemiologic Studies

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Abstract

Analysis of DNA, RNA, and protein extracted from tissue specimens in epidemiologic studies is useful for assessing etiologic heterogeneity, mechanisms of carcinogenesis, and biomarkers for prognosis and prediction of treatment responses. Fresh-frozen tissue samples may provide optimal quality nucleic acids, but pose multiple logistical considerations, including rapid access to tissues before histopathologic examination and specialized equipment for freezing, transport, and storage; in addition, morphology is often compromised. In contrast, formalin-fixed paraffin-embedded (FFPE) tissue samples, including enormous archives of existing specimens, represent a valuable source of retrospective biological material for epidemiologic research, although presenting different limitations compared with frozen samples. Recent efforts have made progress toward enhancing the utility of FFPE specimens for molecular analyses, including DNA studies, and increasingly for RNA and other macromolecules. Here, we report the method that we used to simultaneously recover DNA and RNA from FFPE tissue specimens with appreciable quantity and quality and discuss briefly the application of tumor markers in epidemiologic studies. *Cancer Epidemiol Biomarkers Prev; 19(4); 973–7. ©2010 AACR.*

Tumor Markers and Application in Epidemiologic Studies

Tumor markers, such as genetic mutations, epigenetic changes, and abnormally expressed gene products, are useful to study for the early detection and diagnosis of cancer, determination of treatment strategies, enhancement of prognosis, and etiologic research (1). A key application of tumor markers in epidemiologic studies includes relating tumor alterations to suspected carcinogenic exposures or hereditary factors (in a case-case comparison) and enabling further subspecification of relative risk associations by tumor molecular phenotype to reduce disease misclassification and strengthen causal inference (when case-control comparisons are made). An example among others includes the observation that G: C to T:A transversions at the third base in codon 249 of *P53* are frequently found in hepatocellular carcinoma tissues of patients exposed to aflatoxin, providing important evidence contributing to the establishment of aflatoxin as a carcinogen (2). Tumor markers can also be used in epidemiologic studies to answer many other important research objectives, such as (*a*) the identification of molecular characteristics associated with tumor progression from early lesions to cancer to metastatic disease; (*b*) the identification of molecular characteristics associated with response to treatment, survival, or other prognostic measures; and (*c*) the validation of putative early-disease markers identified through proteomic and metabonomic investigations (3).

DNA from tumor tissue

DNA recovered from tumor tissue can be used to study a variety of genetic events, such as chromosomal instability involving activation of oncogenes and inactivation of tumor-suppressor genes (including copy number changes, translocations, small insertion/deletion events, loss of heterozygosity, and point mutations), or less common genetic alterations such as microsatellite instability, characterized by the presence of mutations in genes with tandemly repeated DNA microsatellite sequences. Tissue DNA can also be used to study epigenetic alterations such as generalized hypomethylation, gene-specific methylation changes in CpG islands of gene promoter regions, and histone modifications.

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Modern high-throughput platforms like comparative genomic hybridization and high-density single-nucleotide polymorphism genotyping arrays offer the ability to characterize large chromosomal alterations such as copy number changes and loss of heterozygosity at high resolution throughout the genome. Genome-wide methylation arrays targeting tens of thousands of genes are available for large-scale investigations of gene-specific methylation changes. An increasing number of studies have successfully shown the utility of DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue for high-throughput assays, showing highly concordant results with DNA isolated from frozen tissue, particularly when the platforms target short fragments of nucleic acids and include high-density coverage of markers (4-7).

RNA from tumor tissue

RNA recovered from tumor tissue can be used to measure gene transcription and expression patterns. Studies to date have largely relied on high-quality RNA from frozen tissue; however, the limited availability of such tissue samples has limited the number and size of such investigations, particularly within population-based epidemiologic studies. Extraction of usable RNA from widely available FFPE tissues has proved problematic. Paraffin embedding, although preserving tissue architecture and proteins, does not preserve nucleic acids very well and can result in RNA degradation. Formalin fixation causes monomethylol additions to the RNA bases and cross-linkage with proteins. Significant efforts have been made to improve the utility of RNA extracted from FFPE tissue by implementing various modifications to the extraction steps (8, 9). Key modifications are complete deparaffinization and reversing of the formalin fixation together with extensive digestion of the FFPE tissue by proteinase K to enable the release of RNA from the cross-linked matrix to maximize the RNA recovery as in fresh tissue. Incubation of RNA in formalin-free buffer (Tris, pH 8.5) at 70°C and extending the incubation period in proteinase K for 3 to 5 days also have been used to remove the monomethylol groups from RNA bases.

However, RNA degradation can occur in the tissue before fixation, and many hospitals do not fix the tissue immediately and appropriately. Although FFPE processing is typically done by the hospitals with little control by the downstream research investigators, a suggestion for a standardized clinical practice is warranted. Studies have established parameters to limit the degradation effects by formalin fixation, including limiting the prefixation time (between surgical incision and fixation) to as short as possible, keeping the fixation time within 12 to 48 hours, and buffering formalin to a neutral pH at 4°C, as reduced pH has been reported to cause degradation of nucleic acids (9).

Despite the challenges, an increasing number of gene expression techniques can be applied to RNA extracted from FFPE. Quantitative real-time PCR is highly sensitive and specific but evaluates a limited number of genes at one time. Microarray technology is generally not as sensitive or specific as quantitative PCR but investigates transcriptional activity on a global scale for biomarker discovery and analysis of patterns of expression. Recent studies comparing RNAs isolated from paired FFPE and frozen samples have shown good performance from FFPE-RNA with valid meaningful results for quantitative real-time PCR assays (10, 11) and even microarrays (11-13), although assays using FFPE-RNA generally had lower sensitivity in detecting transcripts compared with RNA from frozen tissue.

Simultaneous Recovery of DNA and RNA from FFPE Tissue

Numerous commercial kits are available for the extraction of DNA and/or RNA from FFPE tissue samples. Examples of kits used for the extraction of either DNA or RNA include the Epicentre Biotechnologies solution-based digestion/extraction method and the Qiagen QuickExtract column-based capture. RNA extraction kits include the Roche glass fiber fleece capture High Pure and the SuperArray column-based capture ArrayGrade. Kits are also available if both DNA and RNA will be extracted from a FFPE tissue specimen, although most kits require splitting or cutting of the tissue sample into two pieces, one for DNA extraction and one for RNA extraction, resulting in a reduced yield of DNA and RNA and the recovered DNA and RNA derived from different cells. Three commercial kits have been designed to extract both DNA and RNA from the same piece of fresh-frozen tissue (Qiagen multicolumn capture, Allprep) or to extract RNA, DNA, or total nucleic acid (RNA/DNA) from a FFPE tissue specimen (Norgen Biotek column capture, FFPE RNA/DNA Purification kit, or Ambion glass filter method, RecoverAll Total Nucleic Acid Isolation Kit).

Our laboratory at Science Applications International Corporation, National Cancer Institute-Frederick has used preprocessing modifications to the Qiagen Allprep protocol to enable the simultaneous isolation of DNA and RNA from FFPE tissue. The modifications involve using commercially available reagents from Qiagen and others to deparaffinize and reverse the formalin cross-linking and optimizing the duration of these tissue processing steps to maximize the yields of both DNA and RNA (the detailed protocol is provided in Appendix A). We quantitated the extracted DNA using Invitrogen PicoGreen assay, which measures double-strand nucleic acids, and by an absorbance measurement (NanoDrop), which measures single-strand nucleic acids. We also evaluated the extracted DNA for protein contamination by measuring the 260/280 ratio (optimal range, 1.8-2.0), imaging DNA fragmentation on a 1% agarose gel, and for its ability to amplify within sequence-specific PCR reactions. The extracted RNA was quantitated using the Agilent 2100 bioanalyzer system that uses a combination of microfluidics, capillary electrophoresis, and fluorescent dye that binds to nucleic acid to evaluate both RNA

concentration and integrity. We also assessed RNA contamination level by NanoDrop (optimal range, 1.8-2.0). The modified Qiagen method captures RNA sizes greater than or equal to 200 nucleotides (nt), and we measured 28S/18S ratio using the Agilent system to ensure low amount of degraded fragments (optimal range, 1.8-2.0).

We performed extractions on 900 colorectal cancer FFPE tissue cores (1 mm × 3-5 mm for the majority; actual tissue mass smaller than the core size) using the aforementioned protocol. These specimens were collected as a part of a large randomized trial for etiologic and early marker studies, where the researchers had no control over the formalin fixation and paraffin embedding procedures, which were previously completed at various pathology departments all over the United States. We observed an average yield of 9.3 μg DNA (ranging from 3 to 14 μg) and 8.1 μg RNA (ranging from 1 to 19 μg) per tissue core (Table 1). DNA quantitation by PicoGreen yielded a more conservative measure, as opposed to an average yield of 11.6 μg DNA (ranging from 6 to 16 μg) by NanoDrop. Our yields seem to be relatively high on comparison with results from other institutions (14-17), although a direct comparison of DNA/RNA yields between studies is difficult given the many factors that affect yield, such as the specimen size and actual mass of the tissue extracted (e.g., length of the tissue cores, thickness of the tissue sections, and ratio of tissue to paraffin that makes up a core sample), the number of well-preserved cells per sample (e.g., time for fixation and FFPE processing protocol), the tumor type studied, and the extraction protocol used. For example,

in previous studies, 0.33 to 1 μg of DNA was recovered from a 5-μm FFPE tissue section (tumor type not identified; ref. 14) and 8.39 μg DNA/mg of FFPE tissue were isolated from mammary tumors (15), both of which were measured by NanoDrop. RNA recovery by others included 2.55 μg per 20-μm colon control FFPE section (16) and 0 to 3 μg per 5-μm breast tumor FFPE section (17). Our data from samples extracted to date seemed to indicate little to no loss of both DNA and RNA when compared with yields obtained from a straight extraction of either DNA or RNA. Although it would be of interest to evaluate if the quantity of the recovered DNA or RNA was correlated with the storage time (or age) of the tissue specimens, due to the substantial variability in the length and ratio of tissue to paraffin among the FFPE tissue cores, we were unable to objectively evaluate this question.

A sampling of extracted DNA placed on a 1% agarose gel showed fragment sizes between 15 and 30 kb, the 260/280 ratios were in an optimal range between 1.79 and 1.95, and PCR amplifications of specific primers targeting DNA fragment sizes between 35 and 500 bp were successfully tested for 99% of the samples, suggesting that the DNA samples were of good quality (Table 1). All RNA samples had a 260/280 ratio between 1.90 and 2.11 showing minimal contamination, and the fragment sizes were greater than or equal to the 200-nt cutoff as described by the manufacturer with low amounts of degraded fragments shown by 28S/18S ratios ranging between 1.8 and 2.0 (Table 1).

The species of RNA that are not captured by the RNA column that we used are 5.8S rRNA, 5S rRNA, and tRNAs, which together make up approximately 15% to 20% of total RNA. Moving forward, the authors intend to add another isolation to the process that will attempt to isolate the RNA species <200 nt. Additional quality measures will be considered as decisions for downstream applications are being contemplated, including quantitative real-time PCR to more fully characterize the extracted RNA and a full workup and assignment of an RNA Integrity Number on the Agilent system once downstream applications are defined.

Other Issues in the Application of FFPE Tissue Specimens for Epidemiologic Research

Several practical issues should be considered when using FFPE samples in epidemiologic studies. First, given the potential variation in preparation protocol and storage time among FFPE samples in a study, it is useful to perform pilot testing preceding the actual investigation to optimize the extraction protocol, evaluate sample yields and quality, and assess the success rate for applying the recovered DNA and RNA for future assays. Second, RNA tends to be unstable in storage, so the choice of storage buffer and method (e.g., 75% ethanol at −80°C) is critical for optimal long-term usage. To avoid

Table 1. Summary of the extraction results from 900 colorectal cancer FFPE tissue cores

	DNA	RNA
Quantity check		
Mean yield per core, μg	9.3*	8.1[†]
Range of yield per core, μg	3-14*	1-19[†]
Quality check		
Gel imaging fragment size	15-30 kb	≥200 nt
260/280 ratio	1.79-1.95	1.90-2.11
PCR amplification	Successful for 99% of samples[‡]	—

*Measured by Invitrogen PicoGreen assay, quantifying double-strand nucleic acids.
[†]Measured by absorbance (NanoDrop), quantifying single-strand nucleic acids.
[‡]Using specific primers targeting DNA fragments between 35 and 500 bp.

further degradation of RNA due to additional freeze/ thaw steps when aliquoting, it is advisable to plan ahead and distribute the samples in appropriate volumes before long-term storage in freezers. Further, the evaluation of statistical power in the study planning stage should take into account the anticipated rate of success in acquisition of tissue specimens from archives (generally expected to be 50-80%) and the success rate of extracting DNA/ RNA of sufficient quality and quantity for downstream platforms (varying by assays). A comparison between subjects with and without the tissue specimens for study would be useful to assess the generalizability of results.

Conclusion

We have extracted DNA and RNA simultaneously from FFPE tissue specimens with quantity and quality adequate for molecular studies. The wide accessibility of FFPE tissue and the increasing range of applicable analytic methods provide new opportunities in epidemiologic research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Appendix A. Protocol Used for Simultaneous Extraction of DNA, RNA, and Protein from FFPE Tissue Cores

1. Trim the excess paraffin from each core to be extracted using a new Kimwipe tissue for each tissue.
2. Place each trimmed core into appropriately labeled 2-mL snap-cap microfuge tubes. If a sample vial contains multiple cores, all of those cores must be trimmed and recombined into the extraction tube.
3. Within the biosafety cabinet, add 1 mL of Auto Dewaxer to each tube to be extracted.
4. Assure that the core(s) are mixed into the Auto Dewaxer. They will have a tendency to float, so be aware.
5. Close each tube containing the xylene and place into a microfuge and spin at top speed for 2 minutes at 20°C to 25°C. Be sure to control the temperature so as not to fall below 20°C.

6. Carefully remove each tube from the microfuge and open the tubes within the biosafety cabinet.
7. Without touching the tissue, carefully aspirate with a fresh pipette for each tube, the Auto Dewaxer from the tube, and discard into solvent waste.
8. Add 1 mL of Auto Alcohol to each tube, close the caps, and mix each tube by vortexing, making sure that the tissue cores are in contact with the Auto Alcohol.
9. Place each mixed tube into the microfuge and spin at top speed for 2 minutes at 20°C to 25°C.
10. Carefully remove each tube from the microfuge and place in a rack located within a biosafety cabinet. Open each tube once in cabinet.
11. Without touching the tissue, carefully aspirate with a fresh pipette for each tube, the Auto Alcohol from the tube, and discard into liquid waste.
12. Leave the tubes open within the biosafety cabinet for 15 minutes at room temperature to allow the residual alcohol to evaporate fully.
13. Carefully add 24 μL of Qiagen PKD reagent to each tube. Be sure that all of the tissue is in the reagent and not stuck on the side of the tube.
14. Then add 1 μL of Qiagen Proteinase K to each tube, close cap, and mix by vortexing.
15. Using a clean disposable pipette tip for each tube, make sure that the tissue is within the reagent and not stuck on the side of the tube after vortexing.
16. Place each tube in a rack and position the rack in a waterbath at 55°C for 15 minutes. Be sure that the bottom portions of the tubes (to the level containing the reagents) are in the water and that the water never gets close to the tube cap.
17. After 15 minutes at 55°C, remove the rack of tubes from the 55°C waterbath and place the rack in the 80°C waterbath for 15 minutes.
18. Remove the rack of tubes from the 80°C waterbath and allow to cool in the biosafety cabinet to room temperature.
19. Open each tube and add 320 μL of Qiagen RBC reagent. Close the cap of each tube and mix by light vortexing. This is now the lysate tube for each sample.
20. Place an AllPrep DNA spin column into new 2-mL collection tubes from the kit and label the new collection tubes with the appropriate sample ID and "in-process DNA."
21. Place each of the lysate-containing tubes from step #19 into the microfuge and spin at top speed for 3 minutes.
22. Remove the tubes from the microfuge and place in a rack.
23. Carefully open each tube and carefully aspirate the supernatant using a pipette and transfer to the appropriately labeled AllPrep DNA spin column.
24. Once all of the samples have been transferred into the spin columns, place the tubes into the microfuge and centrifuge at 8,000 × g (≥10,000 rpm) for 30 seconds.

25. Remove the tubes from the microfuge and carefully inspect (as well as you can) the columns. The columns should be free of any liquid. If it appears that any of the columns have liquid retained in or on the column, repeat the centrifugation step again.

26. Once all of the liquid is off/out of the DNA columns, carefully remove the DNA column from each tube and place the DNA column into a new, appropriately labeled (with sample ID and "DNA") 2-mL collection tube for later elution of the DNA from the column.

27. Place a new, appropriately labeled RNeasy spin column into a new, appropriately labeled (sample ID and "in-process RNA") 2-mL collection tube.

28. Add 250 µL of 100% ethanol to each of the collection tubes containing the flow-through or effluent from the DNA spin column. This effluent contains the RNA.

29. Mix the tubes well.

30. Transfer the entire volume from the flow-through or effluent tube into the appropriately labeled RNeasy spin column.

31. Add 700 µL of RW1 reagent from the kit to each RNeasy column.

32. Once the RW1 reagent has been added into the RNeasy spin columns, place the 2-mL collection tubes containing the RNeasy spin columns into the microfuge and centrifuge at 8,000 × g (≥10,000 rpm) for 30 seconds.

33. Carefully remove each of the spin columns from their 2-mL collection tubes and transfer the flow-through to a new appropriately labeled 2-mL Eppendorf tube. The label should indicate sample ID and "Protein."

34. Carefully replace the RNeasy spin column back into the appropriately labeled collection tube.

35. Add 500 µL of RPE reagent to each RNeasy spin column (as a wash) and place each tube assembly into the microfuge and centrifuge at 8,000 × g (≥10,000 rpm) for 15 seconds.

36. Add another 500 µL of RPE reagent to each RNeasy spin column and place each tube assembly into the microfuge and centrifuge at 8,000 × g (≥10,000 rpm) for 2 minutes.

37. Remove each RNeasy spin column from their respective collection tubes and place into fresh 2-mL collection tubes labeled with sample ID and "RNA."

38. Add 50 µL of RNase-free water to each RNeasy column.

39. Place each RNeasy tube assembly into the microfuge and centrifuge at 8,000 × g (≥10,000 rpm) for 1 minute.

40. Remove RNeasy column from the 2-mL collection tube and discard the spin column.

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Retraction: Simultaneous Recovery of DNA and RNA from Formalin-Fixed Paraffin-Embedded Tissue and Application in Epidemiologic Studies

The article titled "Simultaneous Recovery of DNA and RNA from Formalin-Fixed Paraffin-Embedded Tissue and Application in Epidemiologic Studies," which was published in the April 2010 issue of *Cancer Epidemiology, Biomarkers & Prevention* (1), is being retracted at the request of the author, Timothy Sheehy, following an investigation conducted by The Office of Research Integrity (ORI) at the U.S. Department of Health and Human Services.

The ORI investigation made a finding of misconduct that affects the main conclusions of this article, related specifically to the data in Table 1, concluding that author Timothy Sheehy "fabricated the quantitative and qualitative data for RNA and DNA purportedly extracted from 900 formalin-fixed, paraffin-embedded (FFPE) colorectal tissue samples presented in Table 1 of the *CEBP* paper and falsely reported successful methodology to simultaneously recover nucleic acids from FFPE tissue specimens, when neither the extractions nor analyses of the FFPE samples were done. Thus, the main conclusions of the *CEBP* paper are based on fabricated data and are false" (2).

No other authors on the article are named in the findings of the ORI investigation of this article.

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