Direct quantification of gene expression in homogenates of formalin-fixed, paraffin-embedded tissues

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BioTechniques 40:481-486 (April 2006)
doi 10.2144/000112133

Formalin-fixed, paraffin-embedded (FFPE) tissues represent an important source of archival materials for gene expression profiling. We report here the development of a modified branch DNA assay that allows direct quantification of messenger RNA (mRNA) transcripts in homogenates from FFPE tissue sections without the need for RNA isolation and reverse transcription into cDNA. Formalin fixation essentially has no effect on the branch DNA assay, and RNA degradation only marginally reduces the signal by 2- to 3-fold. Under the same conditions, formalin fixation and RNA degradation greatly reduces real-time reverse transcription PCR (RT-PCR) efficiency, reducing signals by as much as 15- and 1400-fold, respectively. Although both technologies can generate biologically meaningful expression profiles from FFPE human lung tumor specimens, the branch DNA assay is more sensitive than real-time RT-PCR under the conditions tested. Our results therefore suggest that the branch DNA assay is an ideal tool for retrospective analysis of gene expression in archival tissues.

INTRODUCTION

Formalin-fixed, paraffin-embedded (FFPE) tissue offers a vast source of biopsy specimens for which the clinical outcome is well documented and thus optimal for retrospective studies (1). Over the past decade, FFPE tissue sections have been increasingly used as a source of RNA for gene expression profiling (2–5). However, RNA extracted from FFPE samples has proven to be problematic. First, RNA is significantly degraded in the tissue before, during, and after formalin fixation (6–7). Extended storage also results in more severe RNA deterioration, leading to lower extracted RNA yields. Secondly, the fixed RNA becomes more resistant to extraction due to cross-linking with proteins (2,8). Thirdly, formalin fixation causes mono-methylol addition on all four bases, resulting in a lower efficiency of reverse transcription (8,9). As a result, the current methods of gene expression analysis, such as real-time reverse transcription PCR (RT-PCR), which rely on RNA extraction and reverse transcription, have been consistently less sensitive using archived FFPE tissues than with fresh and frozen tissues (5,10–12).

The branch DNA technology is a sandwich nucleic acid hybridization assay that provides a unique approach for messenger RNA (mRNA) detection and quantification by amplifying the reporter signal rather than target sequences (13–16). By measuring mRNA directly from crude cell lysates and tissue homogenates, the assay avoids variations or errors inherent to extraction and amplification of target sequences. To explore its potential application in archived clinical specimens, we have developed a modified branch DNA assay to quantitatively measure mRNA directly in FFPE tissue homogenates. In this procedure, FFPE sections are treated to remove paraffin, break the cross-linking bonds between RNA and proteins, and lyse the cells to release mRNA. An aliquot of the soluble tissue homogenate is then applied directly to the branch DNA assay. Here we compare the impact of formalin fixation and RNA degradation on the performance of the branch DNA and real-time RT-PCR assays as well as the detection sensitivity measuring mRNA expression in FFPE lung tumor specimens.

MATERIALS AND METHODS

Tissue Homogenate Preparation and Total RNA Isolation from FFPE Tissue Sections

FFPE specimens from 3- and 14-year-old human lung adenocarcinoma and normal adjacent tissues were obtained from Analytical Biological Services, Inc. (Wilmington, DE, USA). Twenty sections of FFPE specimens from the same tissue block were scraped off from glass slides using a scalpel and transferred to a microcentrifuge tube. One milliliter of xylene-containing EZ-DeWax™ (BioGenex, San Ramon, CA, USA) was added to each sample. After briefly vortex mixing and incubating at room temperature for 5 min, the tissue samples were centrifuged in a microcentrifuge at 16,000× *g* for 2 min, and the supernatants were removed. One milliliter 70% ethanol was added to the samples, and the samples were vortex mixed and centrifuged in a microcentrifuge at 16,000× *g* for 2 min. The samples were dewaxed again with the EZ-DeWax and washed with 70% ethanol for two more times before they were divided equally for tissue homogenate preparation or total RNA isolation. To prepare tissue homogenate for the branch DNA assay, 300 µL Homogenizing Solution (Genospectra, Fremont, CA, USA) containing 100 µg proteinase K were added to each dewaxed sample, and the samples were incubated at 65°C overnight. Total RNA was isolated from the dewaxed samples using Optimum™ FFPE RNA Isolation kit (Ambion, Austin, TX, USA). The total RNA was quantified with a SpectraMax® 384 plus (Molecular Device, Sunnyvale, CA, USA).

Formalin Fixation of HeLa Cells

HeLa cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) at an approximate density of 1–2 × 10^6 cells/mL. Ten million HeLa cells were collected, incubated with 10 mL RNA*later*® (Ambion) for 4 h to stabilize the RNA, and washed twice with 50 mL ice-cold 1× phosphate-buffered saline (PBS). The cells were

then incubated with or without 10 mL 1% formalin at 4°C for 1 or 16 h. After being washed twice with 1× PBS, the cells were either lysed with branch DNA Lysis Mixture (Genospectra) or used for RNA isolation with the Optimum FFPE RNA Isolation kit.

RNA Degradation

Fifty micrograms human lung total RNA (Ambion) were treated with 0.1 M NaOH at room temperature. At predetermined time points, an equal volume of 0.1 M HCl was added to the sample to neutralize NaOH and stop RNA degradation. The resulting degraded RNA was used for branch DNA or real-time RT-PCR assays as described below.

Branch DNA Assay

Modified probe design software was developed to design oligonucleotide probe sets for target genes in branch DNA assays (17). A probe set for a target gene consists of three types of oligonucleotide probes (CE, LE, BL) covering a contiguous region of the target, which allows the capture of target RNA to the surface of plate well and hybridization with branched DNA signal amplification molecule. For each target sequence, the software algorithm identifies regions that can serve as annealing templates for CEs (5–10 per gene), LEs (10–20 per gene), or BLs to fill the remaining space. To measure the yield of cDNA reverse transcribed from formalin-fixed or degraded RNA, probes against the antisense strand of the target genes were designed.

The branch DNA assays were performed according to the procedure of QuantiGene® Reagent System (Genospectra), which was previously described in detail (18,19). Briefly, 10 µL tissue homogenate or total RNA were mixed with 40 µL Lysis Mixture (Genospectra), 40 µL Capture Buffer (Genospectra), and 10 µL target gene-specific probe set (CE, 1.65 fmol/µL; LE, 6.6 fmol/µL; BL, 3.3 fmol/µL). Each sample mixture was then dispensed into an individual well of a Capture Plate (Genospectra). To measure cDNA in the QuantiGene assay, the cDNA was mixed with the cDNA strand-specific probe set and heated at 95°C for 10 min before the addition of the Lysis Mixture and Capture Buffer. The Capture Plate was sealed with foil tape and incubated at 53°C for 16–20 h. The hybridization mixture was removed, and the wells were washed three times with 250 µL wash buffer [0.1× saline sodium citrate (SSC), 0.03% lithium lauryl sulfate]. Residual wash buffer was removed by centrifuging the inverted Capture Plate at 340× *g*. Signals for the bound target mRNA were developed by sequential hybridization with branched DNA amplifier and alkaline phosphatase-conjugated label probe at 46°C for 1 h each. Two washes with wash buffer were used to remove unbound material after each hybridization step. Substrate dioxetane was added to the wells and incubated at 46°C for 30 min. Luminescence from each well was measured using a LMax™ Microplate Luminometer (Molecular Devices). Three replicate assays ($n = 3$) were performed for all described experiments.

Reverse Transcription and Real-Time RT-PCR

First-strand cDNA was prepared from purified total RNA using TaqMan® reverse transcription reagents from Applied Biosystems (Foster city, CA, USA) by random priming method. Real-time RT-PCRs were carried out using TaqMan Assay-On-Demand probes and TaqMan Universal PCR Master Mix (Applied Biosystems) in a Mx4000® Real-Time PCR System (Stratagene, La Jolla, CA, USA). The product sizes for β-actin, cyclophilin B, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase A (LDHA), ribosomal protein L19 (RPL19), and ribosomal protein L32 (RPL32) are 171, 67, 122, 130, 127, and 146 bases, respectively. For formalin-fixed HeLa cells and degraded RNA experiments, cDNA was generated from three independent reverse transcription reactions, and each cDNA sample was run in duplicate real-time RT-PCRs. For FFPE lung tumor and adjacent normal tissue samples, only one reverse transcription reaction and duplicate real-time RT-PCRs were performed due to limited RNA yield from normal tissues.

Data Analysis and Statistics

For all samples in the branch DNA assay, background signals were determined in the absence of target mRNAs and subtracted from signals obtained in the presence of target mRNAs. To determine gene expression changes in tumor versus normal tissue in the branch DNA assay, the fold change (FC) was calculated using formula $FC_{bDNA} = (X_T/H_T)/(X_N/H_N)$, where bDNA represents the branch DNA assay, X represents the assay signal from a target gene of interest, H represents the assay signal from a housekeeping gene, T represents the tumor sample, and N represents the adjacent normal tissue sample. For real-time RT-PCR, the comparative cycle threshold (C_T) method was used for data analysis. The ΔC_T is determined by target's average C_T value subtracting the average C_T value of the housekeeping gene. The $\Delta\Delta C_T$ value refers to the tumor sample ΔC_T subtracted by the normal tissue sample ΔC_T. The fold change was calculated as $FC_{qPCR} = 2^{-\Delta\Delta C_T}$, where qPCR represents real-time RT-PCR. Statistical significance was tested using the Student's *t*-test where appropriate ($P < 0.01$).

RESULTS AND DISCUSSION

To examine the effect of formalin fixation on branch DNA and real-time RT-PCR assays, we first treated HeLa cells with RNA*later* to stabilize the RNA and then fixed the cells with 1% phosphate-buffered formalin for 1 or 16 h. The formalin-fixed cells were lysed directly for use in the branch DNA assay, or RNA was isolated and reverse transcribed into cDNA followed by real-time RT-PCR or branch DNA analysis. As shown in Figure 1A, when the branch DNA assay was used to measure mRNA levels in HeLa cell lysates, no significant difference was observed for β-actin, cyclophilin B, and GAPDH between formalin-fixed and unfixed cells. Genomic DNA in the cell lysate does not affect the branch DNA results, since it remains

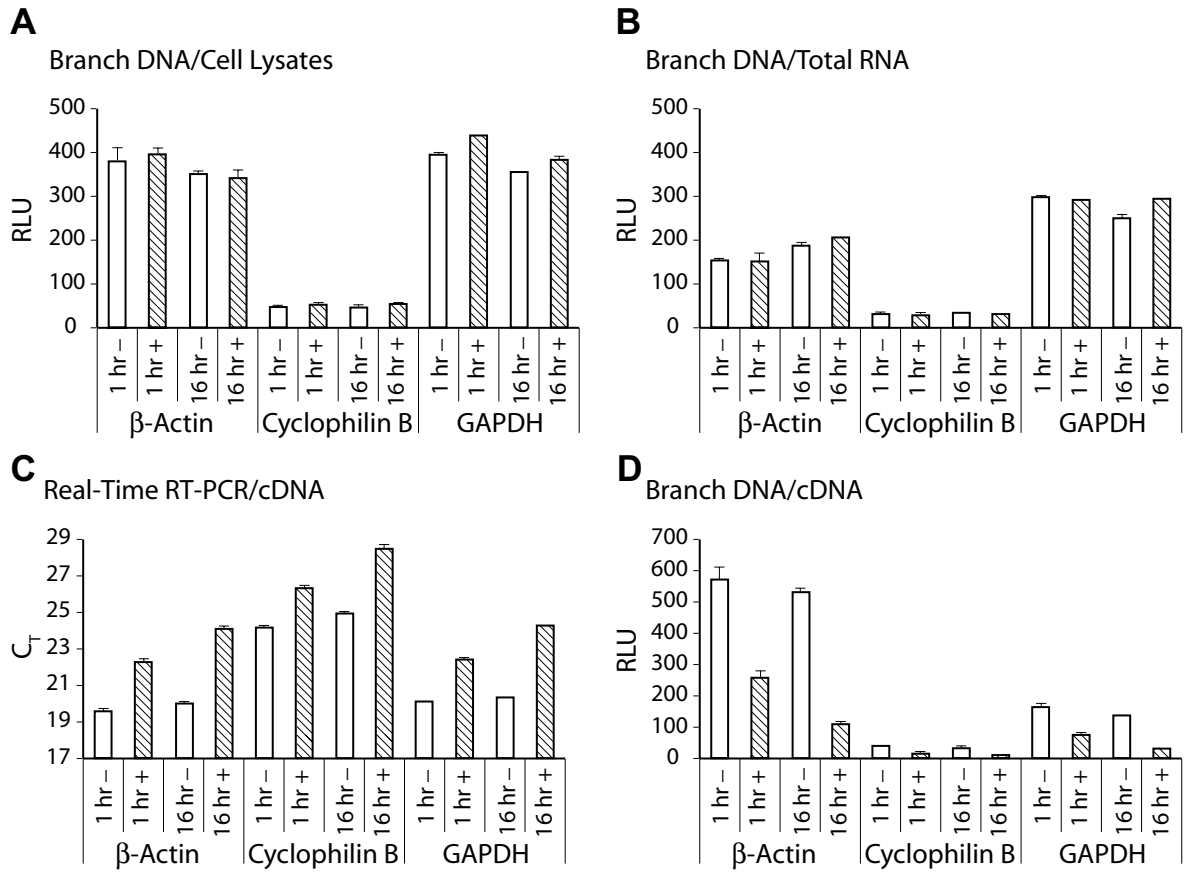

Figure 1. Effect of formalin fixation on the branch DNA and real-time reverse transcription PCR (RT-PCR) assays. Quantification of β-actin, cyclophilin B, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were conducted with the branch DNA assay using cell lysates from 10,000 cells, or approximately 200 ng of equivalent purified total RNA, or cDNA generated from 200 ng total RNA. The assay signal is represented by relative luminescence units (RLU). cDNA generated from 20 ng purified RNA was used in the real-time RT-PCR assay, and assay signal was represented by cycle threshold (C_T) number. An increase of one C_T number represents a 2-fold decrease of starting cDNA templates. (A) Lysates from unfixed (open bar) or formalin-fixed (hatched bar) HeLa cells for 1 or 16 h in branch DNA. (B) Total RNA purified from unfixed (open bar) or formalin-fixed (hatched bar) HeLa cells for 1 or 16 h in branch DNA. (C) cDNA from purified RNA of unfixed (open bar) or formalin-fixed (hatched bar) HeLa cells for 1 or 16 h in real-time RT-PCR. (D) cDNA from purified RNA of unfixed (open bar) or formalin-fixed (hatched bar) HeLa cells for 1 or 16 h in branch DNA. Error bars represents ± one SD.

doubled-stranded throughout the entire procedure and thus cannot hybridize to the branch DNA probe sets at the temperature used in the assay. Similarly, no noticeable difference was observed when the branch DNA assay was used to measure the mRNA levels in total RNA isolated from either fixed or unfixed HeLa cells (Figure 1B). In contrast, using the same RNA samples, prolonged formalin fixation of HeLa cells (1 and 16 h) significantly increased real-time RT-PCR C_T on average by 2.3 and 3.9 C_T, respectively; this corresponds to roughly 5- and 15-fold reduction of starting cDNA templates for real-time RT-PCR amplification (Figure 1C). In order to assess the impact of formalin fixation on the efficiency of reverse transcription of the fixed RNA and conversion to cDNA, we designed cDNA strand-specific probe sets to each of the genes tested in the branch DNA assay. When compared with controls, the signals resulting from the synthesized cDNA on average decreased by 2.4- and 5-fold after fixation for 1 and 16 h, respectively (Figure 1D), indicating that formalin fixation has clearly resulted in the significant reduction in reverse transcription efficiency. This reduction appears to be due to nucleic acid modification alone, not due to RNA degradation, as the sizes of RNA molecules were not appreciably affected by formalin fixation (see Supplementary Figure S1, available online at www.BioTechniques.com).

In genuine FFPE specimens, RNA degradation would be expected to be a serious problem in a significant number of samples. Therefore, we sought to directly test the effect of RNA degradation on the branch DNA and real-time RT-PCR assays. To examine RNA degradation under controlled conditions, we used NaOH treatment to hydrolyze purified RNA, which was then used as input for the two assays. Human lung total RNA was hydrolyzed with 0.1 M NaOH for 3 to 21 min. As shown in Figure 2A, the RNA size decreased with increasing time of RNA exposure to NaOH, as expected. After 21 min of NaOH treatment, the majority of the RNA molecules appeared to be smaller than 200 bp, as determined by gel electrophoresis. We then measured β-actin, cyclophilin B, and GAPDH mRNAs from intact and degraded RNA with branch DNA and real-time RT-PCR assays. As shown in

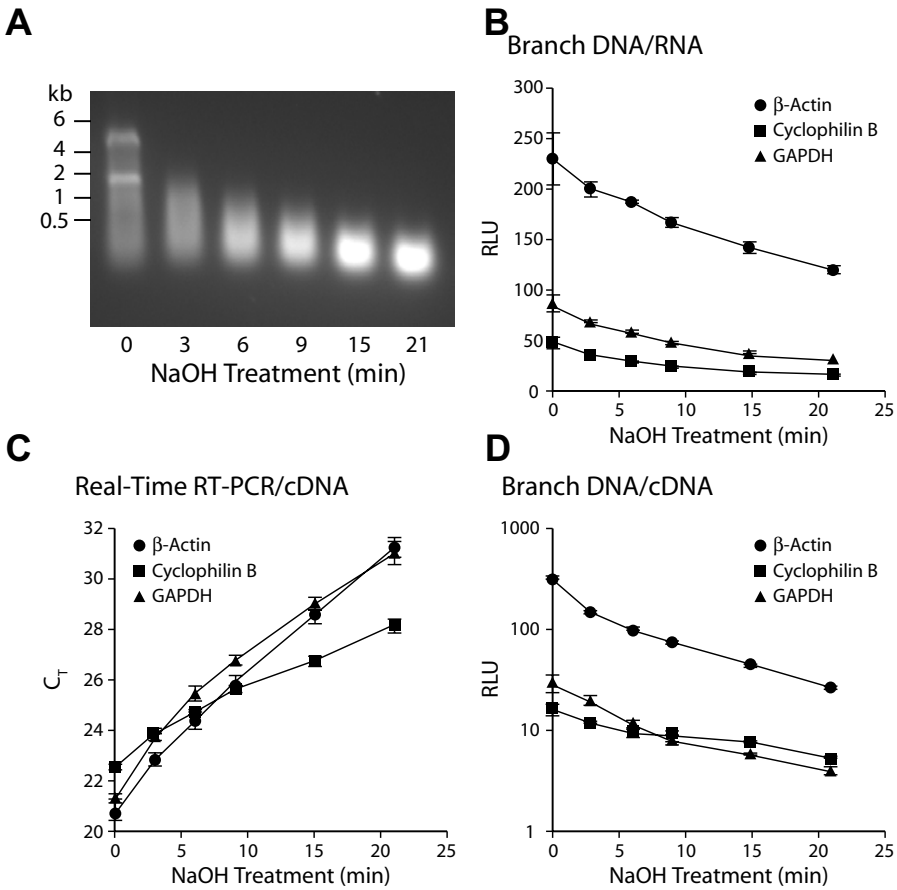

Figure 2. Effect of RNA degradation on branch DNA and real-time reverse transcription PCR (RT-PCR) assays. Quantification of β-actin, cyclophilin B, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were conducted with the branch DNA assay using 200 ng human lung total RNA that were degraded to different extents by NaOH treatment, or using an equivalent amount of cDNA. cDNA generated from 80 ng of the degraded RNA was used in the real-time RT-PCR assay. (A) The size of degraded RNA (1 µg) after NaOH treatment in a 1% agarose gel. (B) Branch DNA assay signals of β-actin (diamond), cyclophilin B (square), and GAPDH (triangle) in RNA treated with NaOH for different period of time. (C) Real-time RT-PCR assay signals and (D) branch DNA assay signals of cDNA generated from the degraded RNA. Error bars represent ± one SD.

Figure 2, B and C, the signals detected for all three genes gradually decreased as the size of the RNA decreased in both assays. However, the drop of assay signals in branch DNA was much more tempered than in real-time RT-PCR. In comparison to the intact RNA, the signal levels for β-actin, cyclophilin B, and GAPDH were gradually decreased by 1.9-, 3.0-, and 2.7-fold, respectively, after 21 min of NaOH treatment (Figure 2B). In contrast, the C_T numbers in real-time RT-PCR assay increased significantly by 10.2, 10.5, and 5 threshold cycles, respectively, which corresponds to a dramatic reduction of starting cDNA templates by 1340-, 1460-, and 50-fold, respectively (Figure 2C). To determine the effect of RNA degradation on reverse transcription and cDNA conversion, we compared the amount of cDNA templates generated from intact or degraded RNA using the branch DNA assay. As shown in Figure 2D, when compared with intact RNA, a 3- to 10-fold signal reduction was observed in the most severely degraded RNA, suggesting that lower reverse transcription efficiency indeed partially contributes to the reduction of real-time RT-PCR performance.

To determine the utility of branch DNA and real-time RT-PCR in gene expression analysis using clinical specimens, we employed FFPE sections from human lung tumor and adjacent normal lung tissue from the same patients. Twenty 10-µm FFPE sections from 3- and 14-year-old lung tumor and matched normal tissue samples were deparaffinated and divided into two halves. One half was lysed, de-cross-linked, and used directly in the branch DNA assay, while the other half was used for RNA isolation and cDNA synthesis, followed by real-time RT-PCR or branch DNA analysis. As can be observed by gel electrophoresis (Figure 3A), the RNA molecules isolated from the 14-year-old FFPE tissues are severely degraded when compared with the 3-year-old samples. To determine the assay sensitivity, we quantified the expression of LDHA and RPL32 in a 4-fold serial dilution of FFPE lung tumor samples. Linear signals were observed for both genes and for both 3- and 14-year-old tumor samples in the branch DNA assay (Figure 3B). Even the smallest amount of sample input (e.g., 1/2560 FFPE section, from which 0.83 ng RNA was extracted) gave quantifiable signals that were four times above the assay background. In comparison, 50 ng of total RNA gave a C_T value of over 35 in the real-time RT-PCR assay in the majority of the samples and genes tested (see Supplementary Table S3). This result suggests that the branch DNA assay appears to be more sensitive than real-time RT-PCR when dealing with FFPE samples.

To determine whether the expression analysis data from FFPE tissues are biologically relevant, we calculated the fold changes of gene expression between lung tumor and adjacent normal tissue of the same patients. RPL19 was used as the housekeeping gene to normalize sample inputs. Normalized LDHA expression in 3-year-old FFPE samples showed approximately 3-fold increase in lung tumor when compared with the adjacent normal tissue as measured by branch DNA and real-time RT-PCR (Figure 3, C and D). This result is consistent with a previously published report (20). We included another control gene, the housekeeping gene RPL32, and normalized its expression to RPL19. The normalized RPL32 expression did not change significantly between the 3-year-old FFPE tumor and the adjacent normal tissue samples for both assays. Therefore, both branch DNA and real-

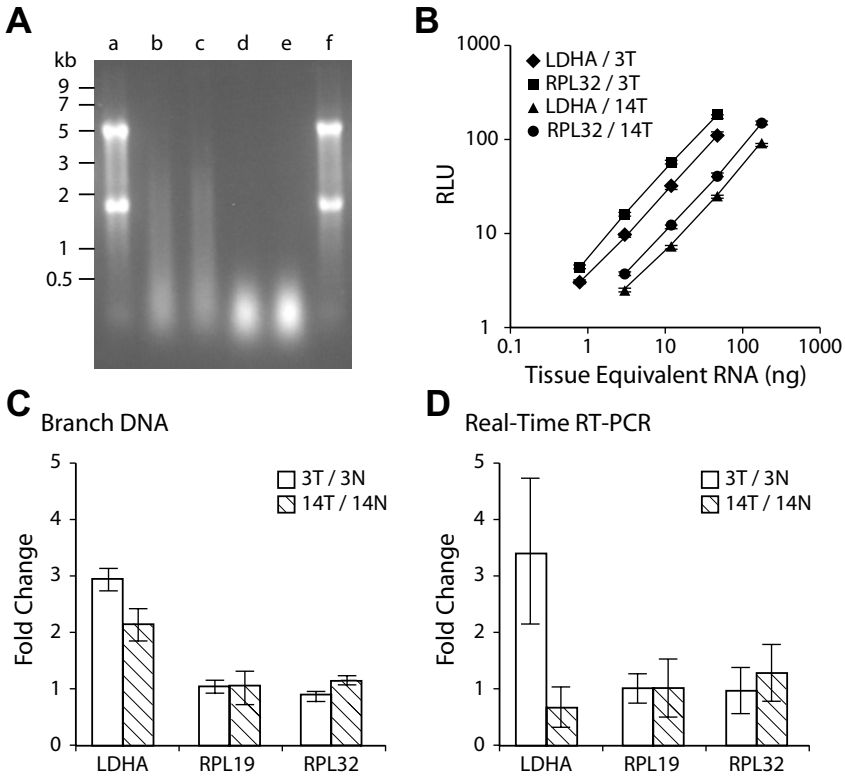

Figure 3. Gene expression profiling in formalin-fixed, paraffin-embedded (FFPE) tissues using branch DNA and real-time reverse transcription PCR (RT-PCR). (A) The quality of RNA isolated from FFPE human lung carcinoma or its adjacent normal tissue was assessed in a 1% agarose gel. The appearances of RNA samples (0.5 µg) in the gel are: lanes a and f, intact human lung RNA as a reference; lane b, 3-year-old tumor (3T); lane c, 3-year-old normal tissue (3N); lane d, 14-year-old tumor (14T); and lane e, 14-year-old normal tissue (14N). (B) Fourfold dilution of homogenates from 3- and 14-year-old tumor sections were measured in branch DNA assays for lactate dehydrogenase A (LDHA), and ribosomal protein L32 (RPL32). (C) Fold change of LDHA and ribosomal protein L19 (RPL19) expression in lung tumor versus adjacent normal tissue normalized against RPL32 was assessed in 3-year-old (open bar) or 14-year-old (hatched bar) FFPE samples as determined by the branch DNA assay. (D) Same as panel C, as determined by the real-time RT-PCR assay.

time RT-PCR can generate biologically meaningful results when the expression data are within the limit of quantification. Furthermore, the normalized LDHA expression of 14-year-old FFPE tumor versus adjacent normal tissue samples showed a significant increase of approximately 2.2-fold using the branch DNA assay. However using real-time RT-PCR, a statistically significant difference between samples was not detected, since the expression data were outside of the reliable measurement range. Interestingly, the signals for the three genes in normal adjacent tissues of 14-year-old samples were 2- to 3-fold lower than in 3-year-old samples in the branch DNA assay (see Supplementary Table S2). In comparison, they were 11- to 26-fold lower in real-time RT-PCR. Thus, in the FFPE samples tested, the branch DNA assay is also less sensitive than real-time RT-PCR to RNA modification and/or degradation.

In summary, the data presented here demonstrate that branch DNA is insensitive to formalin fixation and is much less sensitive to RNA degradation than real-time RT-PCR. As a result, branch DNA is more sensitive in measuring gene expression in FFPE samples. In the experiments described here, both the branch DNA and real-time RT-PCR assays were performed without any optimization. A variety of methods have been attempted to improve real-time RT-PCR detection sensitivity for FFPE samples, ranging from amplification of the purified RNA, using a shorter size product, to using target-specific primers in reverse-transcription (5,21,22). However, these methods do not address the core issues of base modification and RNA degradation in real-time RT-PCR and therefore only lead to relatively modest improvements in assay performance. Given that the experiments presented herein show over 20-fold difference in sensitivity between the branch DNA assay and real-time RT-PCR, even a highly optimized real-time RT-PCR assay would be unlikely to exceed the sensitivity of the branch DNA assay. Moreover, real-time RT-PCR optimization is a time-consuming and labor-intensive process, whereas the branch DNA assay can be used on FFPE samples without special modifications.

We have successfully employed the branch DNA assay to measure gene expression in FFPE tissue sections from human cancer tissues, such as breast, lung, prostate, and colon cancers, and have profiled expression for over 120 target genes from as little as 1/40 of tissue section (data not shown). Recently, a bead-based multiplex branch DNA assay that shares the same assay principle and work flow as the plate-based single-plex branch DNA assay has been described (23); this suggests that, as many as 30 genes can be simultaneously measured directly in homogenates of FFPE tissues. Thus, the simplicity, sensitivity, and reproducibility of the branch DNA assay make it an ideal tool for retrospective analysis of archived tissues, enabling the correlative study of gene expression in response to treatment and clinical outcome.

ACKNOWLEDGMENTS

We would like to thank Wilson Lew for reviewing the manuscript and all members of the Genospectra team for their support.

COMPETING INTERESTS STATEMENT

The authors declare that they are employed by and hold stock in Genospectra, whose product/technology are described in the manuscript.

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Received 16 November 2005; accepted 6 January 2006.

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