Bioanalysis

Evaluation and selection of a non-PCR based technology for improved gene expression profiling from clinical formalin-fixed, paraffin-embedded samples

Aim: Formalin-fixed, paraffin-embedded (FFPE) clinical tissue samples have the potential to provide valuable gene-expression data for the development of cancer biomarkers. However, FFPE RNA is extensively fragmented, presenting a significant challenge for reliably detecting gene expression using traditional qPCR methods. **Results**: We evaluated three novel methodologies along with the traditional qPCR method for their ability to detect Notch pathway gene expression in colorectal cancer FFPE tissue RNAs. We found that quantitative nuclease protection assay-detected gene expression in high-quality RNAs as sensitively as qPCR, and consistently detected mRNAs in highly fragmented FFPE tissue RNAs. **Conclusion**: Quantitative nuclease protection assay represents an improved methodology for detecting gene expression in FFPE tissue and has the potential to advance the development of cancer biomarkers.

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Formalin-fixed, paraffin-embedded (FFPE) tissues are routinely collected for diagnosis, and therefore provide a readily available source of molecular biomarkers for studying the correlation of cancer-associated molecular changes with clinical outcomes. However, the small amount of tissue in biopsy samples limits the number of analyses that can be performed to identify biomarkers that predict therapeutic responses. In addition, nucleic acids, especially RNA, isolated from FFPE tissues are highly fragmented due to their degradation over time. Furthermore, the yield of RNA following traditional extraction procedures is low due to the formalin-induced crosslinking with cellular proteins, and formalin-induced monomethylol addition to the bases, especially adenine, results in reduced reverse transcription (RT) efficiency [1–4]. Although qPCR assays were initially developed to analyze gene expression in FFPE samples [5,6], their sensitivity is often limited by RNA fragmentation.

Recent innovations in gene expression quantification using non-PCR based approaches may provide greater sensitivity for measuring mRNA levels in degraded RNA. Three methods with the potential to address this challenge are the direct multiplexed measurement of nucleic acid with a digital readout (nCounter, Nanostring) [7]; the extraction-free, quantitative nuclease protection assay (qNPA, HTG) [8]; and 3) the branched-DNA signal amplification technology (QuantiGene, Affymetrix Panomics) [9]. These methods decrease the probe size through the use of small hybridization oligomers and/or use sensitive signal amplification/detection methods to improve FFPE sample analysis. To evaluate the potential of these smaller RNA footprint methods for improving the detection of gene expression in FFPE samples, we conducted a pilot evaluation comparing the results of these three novel methodologies with that of qPCR,

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the traditional gold standard for mRNA quantification. For these analyses, we evaluated the Notch pathway gene expression in colorectal tumors, because of the extensive historical data on Notch pathway gene expression in colorectal cancer (CRC) samples [10,11]. Our objective was to identify an improved method that can be used to analyze the gene expression in FFPE samples accurately, for both clinical programs and the development of future diagnostics.

Materials & methods

Assays

To analyze Notch pathway gene expression in CRC samples, eight Notch pathway genes whose expression levels span a range of levels were selected. The Ubiquitin C (UBC) and ribosomal protein LP0 (RPLP0) genes were previously shown to be stable housekeeping genes (HKGs) across CRC patient samples and were selected as HKGs in this study. The probes used in each of the experimental technologies were designed to cover the same transcript regions as analyzed in the TaqMan assays (Applied Biosystems, CA, USA) (Table 1).

Mirrored pairs of fresh frozen (FF) and FFPE tissue blocks from patients with CRC were purchased from Asterand (these specimens were excised from the same tumor section and split in half such that the halves mirror each other). The FFPE RNAs were isolated from consecutive sections using the AllPrep DNA/RNA FFPE Kit (Qiagen, cat#: 80234) following the manufacturer's instructions.

One of the FF RNA samples was artificially degraded (AD) by heating it at 90°C for various time periods. The extent of degradation of all of the RNA samples was assessed by TaqMan RT-qPCR by analyzing the UBC (Hs01871556_s1) gene transcription using the

TaqMan Gene Expression Assay (Hs01871556_s1, Thermo Fisher Scientific), the Agilent Bioanalyzer 2100 and the RNA 6000 Nano Kit (Agilent PN 5067-1511). The extent of degradation was determined by the UBC Ct value, the RNA integrity number and the percentage of fragments greater than 300 nucleotides (DV300), all of which were determined by smear analysis using the Agilent Bioanalyzer 2100 (Tables 2 & 3). RNA samples incubated at 90°C for longer durations exhibited higher Ct values, lower RNA integrity number scores and a lower DV300, consistent with highly fragmented RNA.

Six matched pairs of FF RNAs and FFPE RNAs, along with five AD RNAs exhibiting minimal, intermediate and high levels of degradation, and the corresponding control FF RNAs were selected for evaluation. These 18 RNA samples were assigned to two groups: intact RNAs and the fragmented RNAs. The intact RNAs (n = 7) consisted of the six FF RNAs and the AD control RNA, and the fragmented RNAs consisted of the six FFPE RNAs and the five AD RNAs. The RNA samples were randomized, and aliquots from the same extracted RNA sample were distributed to four different assay laboratories for analysis. In addition, six matched FFPE tissue samples were used directly for the qNPA and QuantiGene platform analysis without RNA extraction. These samples were prepared from sections adjacent to those used for RNA extraction.

TaqMan RT-qPCR

All of the TaqMan assays were ordered from LifeTech Assay-on-Demand (Life Technologies, CA, USA) and were either inventoried or made-to-order assays. Individual RT-qPCR assays were performed to quantify the expression of each mRNA, and the data

Gene Name	Gene type	TaqMan assay ID	Refseq ID	Exon boundary	Assay location	TaqMan amplicon (bp)
Notch1 3′ end	Target	Hs01062014_m1	NM_017617.3	33–34	6175	80
Notch1 TM	Target	Hs00413209_g1	NM_017617.3	28–29	5385	92
Notch1 5′ end	Target	Hs01062012_m1	NM_017617.3	2–3	137	110
HES1	Target	AIT9549	NM_005524	3–4	521	79
HES4	Target	Hs00368353_g1	NM_001142467.1	2–3	572	97
HEY2	Target	Hs01012057_m1	NM_012259.2	3–4	439	90
DLL4	Target	Hs00184092_m1	NM_019074.3	6–7	1126	78
Numb	Target	Hs01105433_m1	NM_001005744.1	10–11	1271	71
UBC	HKG	Hs01871556_s1	M26880.1	N/A	2173	135
RPLP0	HKG	RPLP0_4333761	NM_053275.3	3–3	N/A	105

Table 1. List of Notch pathway and housekeeping genes and TaqMan assay design.

The Notch1 TaqMan assays were designed to monitor transcription at the 5′ and 3′ ends, and at the transmembrane domain.

were analyzed using the ΔCt relative quantification method. All of the assays, which were run in-house, exhibited efficient PCR amplification. The optimal RNA input (20 ng per reaction) was determined by analyzing a series of linearly diluted RNAs (from 80 to 0.63 ng).

In brief, 1 µg of total RNA was reverse transcribed to cDNA in a total volume of 50 µl using the ABI High Capacity RNA-to-cDNA Kit (Life Technologies, P/N: 2387406) following the manufacturer's instructions. For the amplification, the cDNA was diluted 1:1 in water to a concentration of 10 ng/µl. Each qPCR reaction was prepared by adding 2 µl of cDNA to 10 µl of 2× TaqMan Gene Expression Master Mix (LifeTechnologies, P/N: 4369016) and 1 µl of 20× TaqMan Gene Expression Assay Buffer in a final volume of 20 µl. Each sample was run in triplicate in a 384-well plate on a LifeTechnologies ViiA 7 instrument using the ABI standard protocol and default settings [12]. One QC sample was added to each qPCR run to monitor interassay variability.

The data were analyzed and exported to a Microsoft Excel file. The Ct values of each of the target genes and the HKGs (UBC and RPLPO) were calculated, and then the ΔCt value for each of the target genes was calculated using the average Ct of the HKGs. The $\Delta\Delta$Ct for each of the target genes in the paired FF and FFPE RNAs was assessed by calculating the ΔCt difference between the two samples.

HTG qNPA plate assay

The qNPA plate assay was used to analyze both FFPE tissue and RNA samples. The assay contained probes for eight Notch pathway genes that were designed and developed by HTG Molecular Diagnostics, Inc. (AZ, USA), and probes for a negative control plant gene (ANT) and positive process controls. The samples were run on the HTG Edge System, which con-

sists of an automated liquid handling unit (Processor) and a plate imaging unit (Reader) [13].

The FFPE lysates were prepared using an HTG Edge Sample Prep Reagent Kit. The FFPE tissue was scraped from a glass slide into a 1.5-ml microfuge tube and lysed with HTG lysis buffer so that 25 µl of lysis buffer contained 0.3 cm^2 of FFPE tissue. Denaturation oil (500 µl) was layered on top of the lysis buffer, and the samples were incubated at 95°C for 15 min. To improve tissue lysis, the samples were treated with Proteinase K at 1/20th the volume of the lysis buffer and were incubated at 50°C for 60 min. The FFPE lysates were analyzed along with the intact and fragmented RNA samples, which were directly diluted in lysis buffer and run at 200 ng/well. Human universal RNA (Agilent cat# 740000) was used as a process control at 100 ng/well to monitor interplate variability. Approximately 25 µl of each sample was transferred to

Table 2. Degradation grades of the five artificially degraded RNAs and the fresh frozen RNA control.

FF RNAs	AD RNAs (degradation grade)	TaqMan (Ct_UBC)	RIN	DV300 score (%)
FF RNA-7	Control	22.82	9.3	93
	AD grade 1	25.27	2.3	56
	AD grade 2	30.42	2.0	7
	AD grade 3	33.24	2.6	7
	AD grade 4	35.46	2.6	5
	AD grade 5	38.77	1.6	8

Degradation grades were determined by UBC TaqMan Ct values, RIN and the percentage of RNA fragments greater than 300 nucleotides (DV300 scores). The degradation scores are from low to high: 1–5 (5 representing the highest degradation level), and are relative to that of the FF RNA control.
AD: Artificially degraded; FF: Fresh frozen; RIN: RNA integrity number.

Table 3. Six paired formalin-fixed, paraffin-embedded tissues and fresh frozen/formalin-fixed, paraffin-embedded RNAs.

FFPE tissue	FF RNAs	FFPE RNAs (degradation grade)	TaqMan (Ct_UBC)	RIN	DV300 (%)
FFPE slides-1	FF RNA-1	FFPE RNA-1 (grade 1)	25.96	2.2	53
FFPE slides-5	FF RNA-5	FFPE RNA-5 (grade 2)	27.03	2.1	39
FFPE slides-4	FF RNA-4	FFPE RNA-4 (grade 3)	28.23	2.5	88
FFPE slides-2	FF RNA-2	FFPE RNA-2 (grade 4)	32.06	2.1	26
FFPE slides-6	FF RNA-6	FFPE RNA-6 (grade 5)	33.24	2.3	36
FFPE slides-3	FF RNA-3	FFPE RNA-3 (grade 6)	37.31	2.5	4

Relative degradation grades were determined as described in **Table 2**. RIN, percentage of RNA fragments greater than 300 nucleotides (DV300). Degradation scores are from low to high: 1–6 (6 representing the highest degradation level).
FF: Fresh frozen; FFPE: Formalin fixed, paraffin embedded; RIN: RNA integrity number.

a well in a 96-well sample plate. All samples were run in triplicate on the HTG Edge System.

Three forms of data were generated from the Edge System (raw, filtered raw and normalized), and then exported to a Microsoft Excel file. The filtered-raw data were background-subtracted raw data that were filtered to remove the data from failed wells or outliers. The normalized data were filtered-raw data corresponding to target genes that were normalized to the data of the HKGs to minimize the effects of technical variation. The average normalized value of the technical triplicates was calculated and used for data analysis.

NanoString nCounter gene expression assay

NanoString's nCounter technology is based on the digital detection and direct molecular barcoding of target molecules through the use of a color-coded probe pair. The nCounter Expression Assay is run on the nCounter System, which consists of two instruments, the nCounter Prep Station used for posthybridization processing and the Digital Analyzer used for data collection.

A Notch Custom CodeSet was developed and validated by NanoString Technologies, Inc. (WA, USA). The CodeSet consists of reporter and capture probes that hybridize to the target sequences of interest, forming a tripartite complex. The nCounter assay analyzing our RNA samples was run at NanoString following the nCounter Gene Expression Assay User Manual [14]. The hybridization reaction was prepared by transferring 10 μl of Reporter CodeSet, 10 μl of hybridization buffer, 5 μl of sample RNA (200 ng RNA) and 5 μl of Capture ProbeSet (in that order) into 12-strip PCR tubes. The strip tubes were immediately placed in a preheated thermocycler programmed to remain at 65°C. After a 16-h incubation, the strip tubes were immediately moved to the nCounter Prep Station, where the samples were subjected to a 3-h automated procedure in which the tripartite complexes were purified and immobilized on a cartridge for data collection. The digital images were processed on the nCounter Digital Analyzer, and the raw data were exported in reporter code count files. The reporter code count files were analyzed using the NanoString data analysis software, nSolver®. The raw counts, normalized counts and fold changes in mRNA detection were generated by nSolver.

QuantiGene multiplex assay

The QuantiGene Multiplex 2.0 assay is a combination of branched DNA signal amplification and multianalyte profiling bead (xMAP®) technologies that enables the detection and quantification of multiple RNA tar-

gets simultaneously. A multiplex assay consisting of the eight Notch pathway genes and two HKGs was custom-designed using TaqMan assay region-matched probes, and the assays were carried out following the QuantiGene Plex user manual (Affymetrix, CA, USA). Briefly, the matched FF and FFPE tissue RNAs (240 ng per reaction), the AD RNAs (240 ng per reaction) and the FFPE tissue homogenates (one slide for two reactions) were hybridized with the QuantiGene 10-plex probeset. Approximately 60 μl of working bead mix (containing lysis mixture, Proteinase K, blocking reagent, probeset and bead set) was dispensed into each well of the hybridization plate, and then 40 μl of lysate or diluted lysate was added to each well. The hybridization plate was sealed with a pressure seal and then incubated for 18 h at 54°C with shaking at 600 r.p.m. (VorTemp™ 56 Shaking Incubator, Labnet International, Inc.).

The next day, the wash solution, preamplifier solution, amplifier solution, labeled probe and streptavidin-phycoerythrin (SAPE) solution were prepared according to the manufacturer's recommendations. The hybridized RNAs were transferred to a magnetic separation plate and washed three-times. The signal was amplified by sequential hybridizations with the preamplifier and amplifier, followed by hybridization with a labeled probe. Each of the incubations was performed for 1 h at 50°C and was followed by a wash cycle. The SAPE solution was added, and the plate was incubated in the dark at room temperature for 30 min. The unbound SAPE was washed away using SAPE wash buffer, and then 130 μl of SAPE wash buffer was added to each well followed by shaking at room temperature for 3 min to resuspend the beads. The plate was then read using a Flexmap 3D instrument (Luminex Corporation).

The fluorescent reading generated from the Flexmap 3D represents the raw data. The fluorescent values for each mRNA of interest were background-subtracted using the blank well reads. Those values exceeding the background were normalized to the geometric mean of the signal derived from the two HKGs in each sample. The normalized values were then used for data analysis.

Data analysis

The correlation and expression data were statistically processed with TIBCO Spotfire (TIBCO Spotfire, Inc.). The correlation between fold change and normalized data was determined using linear regression and expressed as the R^2 coefficient. Suitable reference genes (RGs) for the normalization of gene expression data were evaluated by stability and variability testing using the Normfinder algorithm. The geometric mean of two RGs was used as a reference index.

Results

Comparison of the gene expression data generated from high-quality RNAs using the four technologies

To confirm that each platform performed well when high-quality RNAs were used, the six FF RNAs were subjected to analysis with the TaqMan qPCR, NanoString nCounter, HTG qNPA and Panomics Affymetrix QuantiGene platforms. The gene expression values for the eight Notch pathway genes were normalized against the geometric mean of the HKGs, and then the correlations between the normalized gene expression values obtained from each of the three non-PCR technologies and that obtained from TaqMan were analyzed (Figure 1A). The data obtained using qNPA exhibited the highest average correlation, followed by that obtained using the QuantiGene multiplex. The data obtained using nCounter exhibited the lowest correlation with the TaqMan data ($R^2 < 0.4$). Next, we calculated a fold-change value for each platform by comparing the normalized gene expression values obtained in FF RNAs 2–5 with those obtained from FF RNA-1. We found that the fold changes from the non-PCR platforms correlated well with the TaqMan fold changes (Figure 1B) suggesting that the four technologies performed equally well in measuring relative gene expression when high-quality RNAs were analyzed.

Sensitivity of gene expression detection in AD & FFPE RNAs

To qualitatively assess the effect of RNA degradation/fragmentation on each platform's detec-

tion capability, we analyzed Notch pathway gene expression levels in the AD and FFPE RNAs using different platforms, and counted the number of genes that were detected in the samples. Analysis of the AD RNAs (Table 4) showed that QuantiGene and HTG qNPA performed better than nCounter and TaqMan in detecting gene expression in highly fragmented samples. QuantiGene and HTG qNPA were also more capable than nCounter and TaqMan in detecting gene expression in the highly fragmented FFPE RNAs (Table 4).

Detection of Housekeeping gene expression in AD RNAs

To quantitatively assess the effect of RNA degradation/fragmentation on each platform's detection capability, we examined the transcript levels of two HKGs (UBC and RPLP0) with each platform. We plotted the HKG signals as a function of degradation grade and found that the signals detected by all four platforms dropped with increasing degradation (Figure 2). However, the TaqMan qPCR curve had the steepest slope, followed by that of the QGM curve, and both were steeper than that of the nCounter and qNPA curves. Notably, the qNPA curve was almost flat, suggesting that this assay was relatively resistant to RNA fragmentation. Our data also suggested that shorter amplicons or probe sequences may correlate with increased mRNA detection in fragmented RNA samples, since the qNPA platform uniformly uses 50-nucleotide nuclease protection probes, which is the
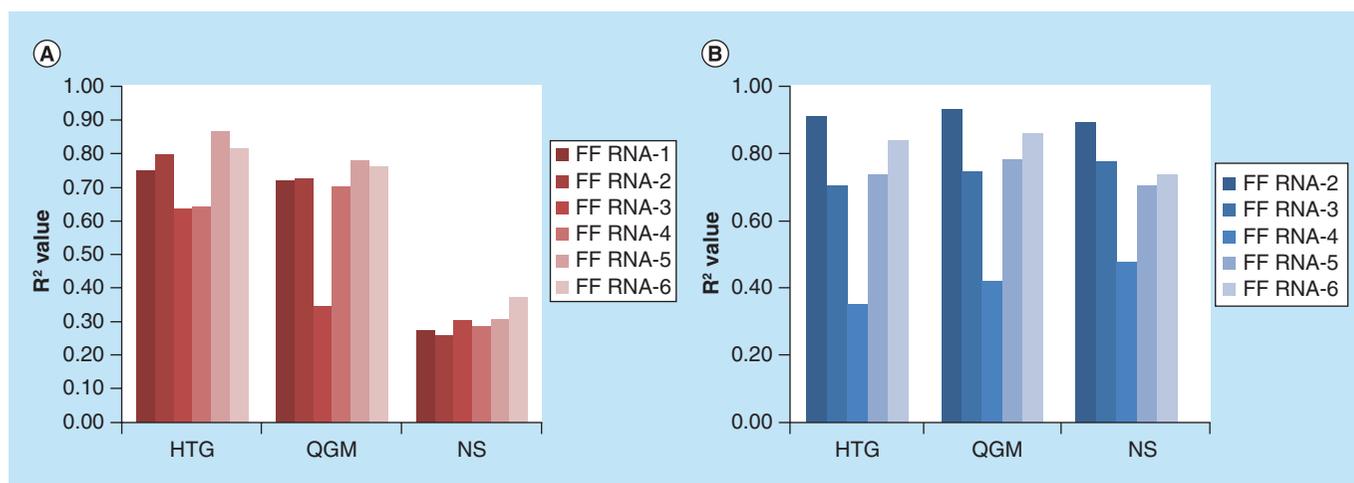
Figure 1. Correlation of normalized gene expression levels and fold changes of high-quality RNAs obtained using TaqMan and three novel technologies. (A) Normalized Notch pathway gene expression was analyzed for six FF RNA samples using each technology. The correlation between the normalized gene expression values obtained from the three novel technologies and that obtained using TaqMan was determined by linear regression. **(B)** Fold changes in gene expression between FF RNA-1 and the other RNAs (FF RNAs 2–6) were determined for each of the technologies, and then the linear regression coefficients (R^2 values) were calculated between the TaqMan fold changes and that of the three novel technologies. Four technology formats were used: HTG, QGM, NS and TaqMan. The resulting R^2 values were plotted for comparison.
FF: Fresh frozen; HTG: HTG qNPA; NS: NanoString nCounter; QGM: QuantiGene.

shortest probe length among the four platforms, while the TaqMan qPCR assay generated relatively long amplicons, the size of the UBC amplicon being 135 bp.

Correlation of Notch pathway gene expression in the intact & fragmented RNAs using the four platforms

To assess the ability of each technology platform to detect and quantify gene expression in degraded/fragmented RNAs, we analyzed the correlation between the Notch pathway gene expression signals obtained from the control RNA and those obtained from the AD samples using the four technologies (Figure 3A), and then ranked the R^2 coefficients. The ranked order of the R^2 coefficients was HTG qNPA > TaqMan > QuantiGene ≥ nCounter. We also derived correlation coefficients for each of the paired FFPE and FF RNAs (Figure 3B), and found that the ranked order of the R^2 coefficients was HTG qNPA > nCounter > QuantiGene > TaqMan. In summary, the HTG qNPA platform consistently performed better than the other technologies in detecting and quantifying gene expression in the fragmented RNAs.

Correlation of Notch pathway gene expression in matched FFPE tissue lysates & RNAs using QuantiGene & HTG qNPA

Since QuantiGene and HTG qNPA can directly detect RNAs in tissue lysates, we assessed the ability of these two platforms to detect and quantify gene expression in FFPE tissue lysates. We derived the gene expression cor-relation coefficients for FFPE tissue lysates versus their matched FFPE RNAs, FFPE tissue lysates versus their matched FF RNAs and FF RNAs versus their matched FFPE RNAs for each technology (Figure 4). Both tech-nologies generated highly correlated gene expression data from the FFPE tissue lysates and FFPE RNAs. Notably, however, the data generated by HTG qNPA exhibited stronger correlations between the FFPE tissue lysates and FF RNAs, and between the FF and FFPE RNAs than the data generated by QuantiGene.
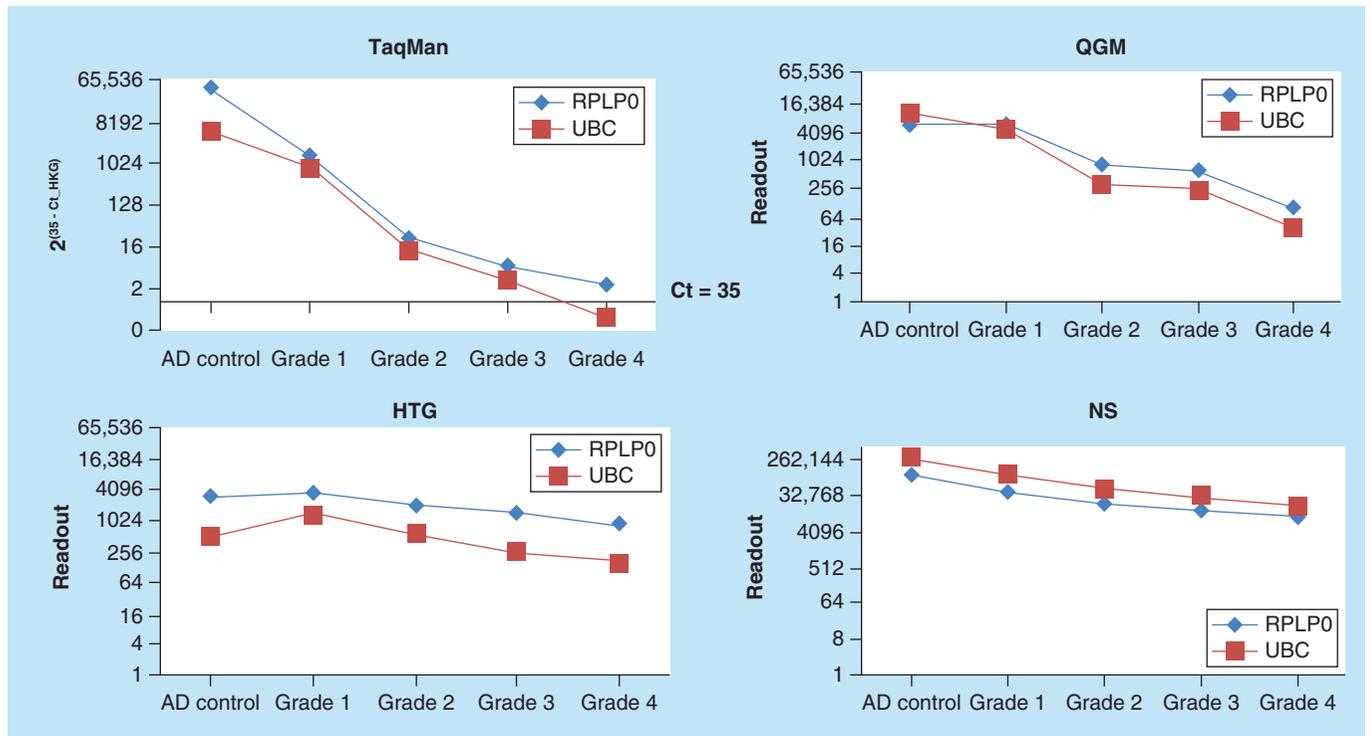
Analysis of fold changes between the detected gene expression in the AD RNAs & the control RNA

To further assess the ability of each platform to detect and quantify gene expression change (fold change) in the AD RNAs, we analyzed the Notch pathway and HK gene expressions detected in the control and AD sam-ples using the four technologies, and then examined the fold changes in the detected expression levels of each of the ten genes in the AD samples, compared with that of the control. A fold change of 1.0 would indicate that the assay platform could reliably detect and quantify the mRNAs in degraded samples. Here, we observed vari-able fold changes in the expression data generated by TaqMan, nCounter and QuantiGene, with the TaqMan data exhibiting the highest fold changes (Figure 5A). In contrast, the HTG qNPA-derived expression data exhib-ited the least variability between the AD and control RNA samples.

Sample	TaqMan	HTG	QGM	NanoString
Table 4. Gene expression detection sensitivity in artificially degraded RNAs and formalin-fixed, paraffin-embedded RNAs.				
Control	All	All	All	All
AD grade 1	All	All	All	All
AD grade 2	All	All	All	1 undetected
AD grade 3	1 undetected	1 undetected	All	1 undetected
AD grade 4	2 undetected	1 undetected	All	1 undetected
AD grade 5	10 undetected	4 undetected	4 undetected	9 undetected
FFPE RNA grade 1	All	All	All	All
FFPE RNA grade 2	All	All	All	1 undetected
FFPE RNA grade 3	All	All	All	All
FFPE RNA grade 4	All	All	All	All
FFPE RNA grade 5	2 undetected	All	All	1 undetected
FFPE RNA grade 6	10 undetected	4 undetected	5 undetected	9 undetected
Detection criteria	Ct < 38	signal > bkgd	signal > bkgd	signal > bkgd

Notch pathway and housekeeping gene expressions were analyzed in one control, five AD RNAs (from grade 1 to grade 5, with grade 5 being the most fragmented) and six FFPE RNAs (from grade 1 to grade 6, with grade 6 being the most fragmented) using the four different technologies: HTG, QGM, NS and TaqMan.
AD: Artificially degraded; All: All ten genes detectable; bkgd: Background; FFPE: Formalin fixed, paraffin embedded; HTG: HTG qNPA; NS: NanoString nCounter; QGM: QuantiGene.

Figure 2. Housekeeping gene mRNA detection in artificially degraded RNAs. The detection of two HKG mRNAs (RPLP0 and UBC) was analyzed in four AD RNAs (from AD grade 1 to AD grade 4, with grade 4 being the most fragmented) using four technology formats: TaqMan, HTG, QGM and NS. The TaqMan readouts (antilog2 of -Ct value) and readouts from the other platforms were plotted as a function of degradation grade.

AD: Artificially degraded; HTG: HTG qNPA; NS: NanoString nCounter; QGM: QuantiGene.

Analysis of fold changes between the detected gene expression in the paired FF & FFPE RNAs

To further assess the ability of each platform to detect gene expression change (fold change) in the naturally degraded FFPE RNAs, we analyzed the gene expression in each of the FFPE and FF RNA pairs detected by each of the platforms, and then calculated the fold change for each of the ten genes. Similar to our analysis of the AD and control RNAs, we observed variable fold changes in the detected gene expression derived from TaqMan, NanoString and QuantiGene, with the data from TaqMan showing the highest variability, while the expression data generated from by HTG qNPA exhibited the least variability between the FF and FFPE RNAs (Figure 5B).

Discussion

Here, we examined the performance of three non-PCR technology platforms (nCounter, QuantiGene and HTG qNPA) in detecting and quantifying the gene expression in AD RNA samples or in FFPE samples, and compared the results with that of TaqMan qPCR. Our findings indicated that qNPA performed better than the others in detecting mRNAs in degraded samples and that the size of the probe or amplicon plays a significant role in the detection of

mRNAs in these samples. Among the four technologies, HTG qNPA has the smallest probe footprint (50 bases); nCounter has a 100-base footprint (the combination of the capture probe and reporter probe); and QuantiGene, which uses multiple short probes, has a hybridization footprint that ranges between 50 and 100 bases. In contrast, qPCR generates amplicons that are typically 70–200 bases, with the PCR amplicon size ranging from 71 to 135 bases in this study. Thus, the probe/amplicon size appears to be inversely proportional to the mRNA detection sensitivity in fragmented samples.

Our findings also suggest that the use of probes or amplicons with uniform sizes may also contribute to the accuracy of gene expression analysis in fragmented samples. The qNPA, nCounter and QuantiGene platforms use uniform probe sizes for all genes, while qPCR amplicons vary in length. Differences in amplicon size can also affect the normalization of gene expression, which is based on housekeeping gene expression. Thus, we conclude that inconsistent amplicon size may have led to the large fold changes in gene expression between the intact and fragmented RNAs observed in the TaqMan analysis (Figure 5).

Another advantage of both the qNPA and Quanti-Gene platforms is that neither requires RNA extrac-
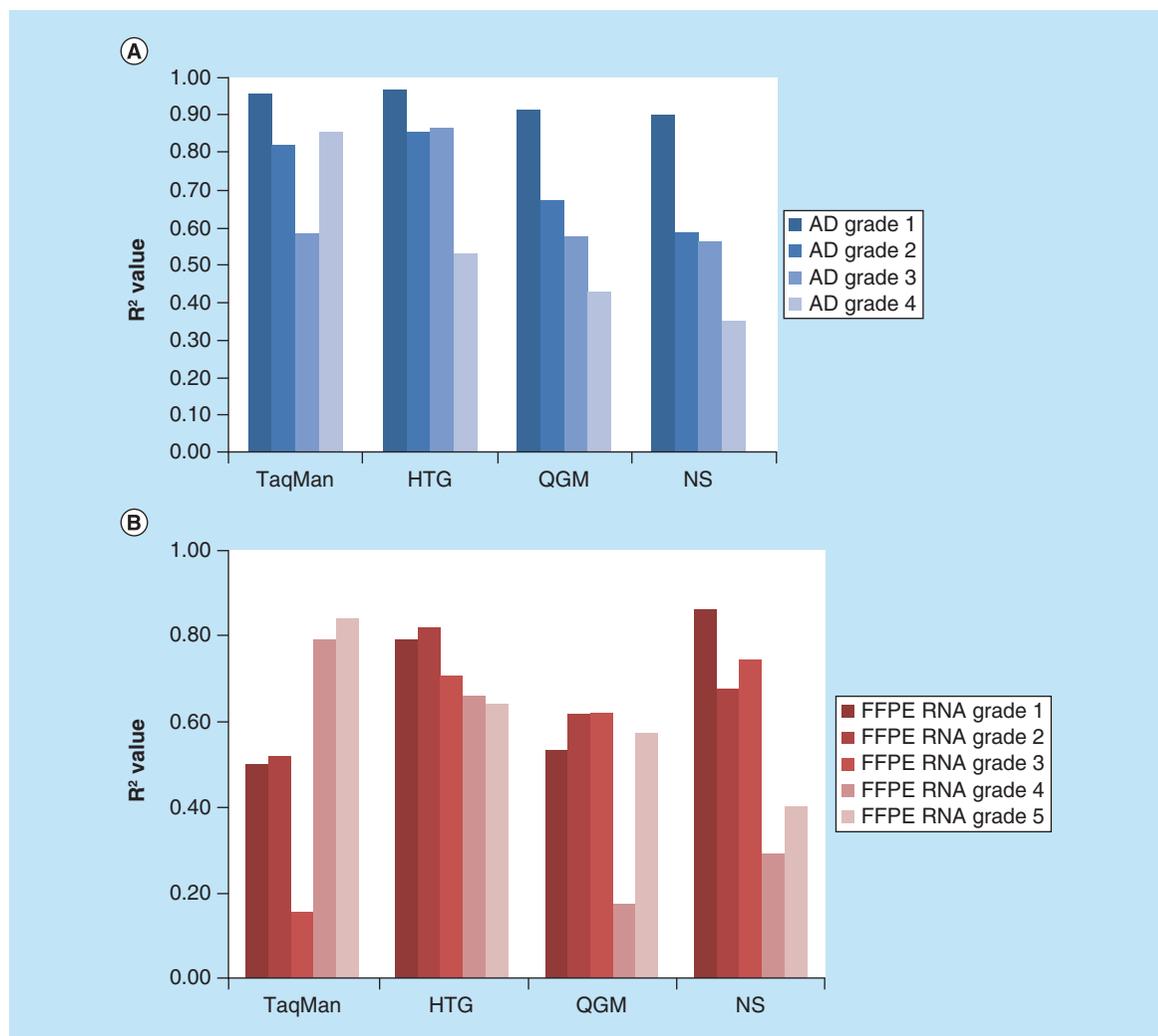
Figure 3. Correlation of normalized gene expression between control and artificially degraded RNAs and between matched fresh frozen and formalin-fixed, paraffin-embedded RNAs using the four technologies.
(A) Notch pathway and HK gene expressions were analyzed in the control and four AD RNAs (from AD grade 1 to AD grade 4, with grade 4 being the most fragmented) using the four different platforms. The correlation between the normalized gene expression values from the four AD RNAs and that from the control RNA was analyzed for each technology. **(B)** Notch pathway and HK gene expression was analyzed in five matched FF and FFPE RNAs (FFPE RNAs from grade 1 to grade 5, with grade 5 being the most fragmented) using four platforms. The correlation between the normalized gene expression values from the FF RNAs and those from the FFPE RNAs was determined for each technology. Four technology platforms were used: HTG, QGM, NS and TaqMan. A plot showing the R^2 values obtained for each platform is shown.
AD: Artificially degraded; FFPE: Formalin fixed, paraffin embedded; HTG: HTG qNPA; NS: NanoString nCounter; QGM: QuantiGene.

tion. Nucleic acid extraction from FFPE requires harsh conditions, which can lead to further RNA degradation. FFPE tissue lysate analysis using the qNPA and QuantiGene platforms led to more consistent mRNA detection than the analysis of FFPE RNA in these assays **(Figure 4)**. In fact, one RNA sample extracted from FFPE was not able to yield meaningful results by NanoString or QuantiGene, but HTG yielded a result that agreed well with the gene expression of the corresponding frozen sample. Furthermore, the gene expression in the FFPE samples was more highly correlated with that in the matched FF RNAs when the FFPE lysate, rather than the RNA was used. The increased detection of mRNAs in the RNA extraction-free procedures may be due to the reduced loss of RNA.

TaqMan qPCR can produce data with a dynamic range of seven to eight log orders of magnitude [15]; HTG qNPA has a linear dynamic range over three orders of magnitude [8,16–17]; and the nCounter system response is linear over more than two orders of magnitude [7,18]. Thus, the dynamic range of detection of the non-PCR based methods is not as large as that of the gold standard

method; this is a potential limitation even for FFPE gene expression analysis, and more study is needed to address this gap. Notably, new methods are being developed to increase the linear dynamic range of HTG qNPA (see the 'Future perspective' section).

Another issue is the data repeatability and reproducibility of each method. The reported CVs for TaqMan qPCR are low, approximately 24% [15]. Bourzaca et al. [16,19] reported that the Edge platform CVs between replicate wells averaged approximately 15% or less. In addition, we found that in cases where the same tumor FFPE samples were run in similar experiments at HTG, and in our laboratory, the CVs between

interlaboratory runs averaged approximately 25% or less (data not shown).

Conclusion

Here we designed comprehensive experiments to evaluate the performance of three novel non-PCR technologies in measuring mRNA expression in FFPE tissue-derived RNAs. The HTG qNPA platform detected the gene expression in high-quality RNAs as sensitively as TaqMan, and maintained its ability to detect mRNAs in both AD RNA samples and FFPE tissue samples. Furthermore, HTG qNPA can be used to measure gene expression in FFPE lysates without the need for RNA
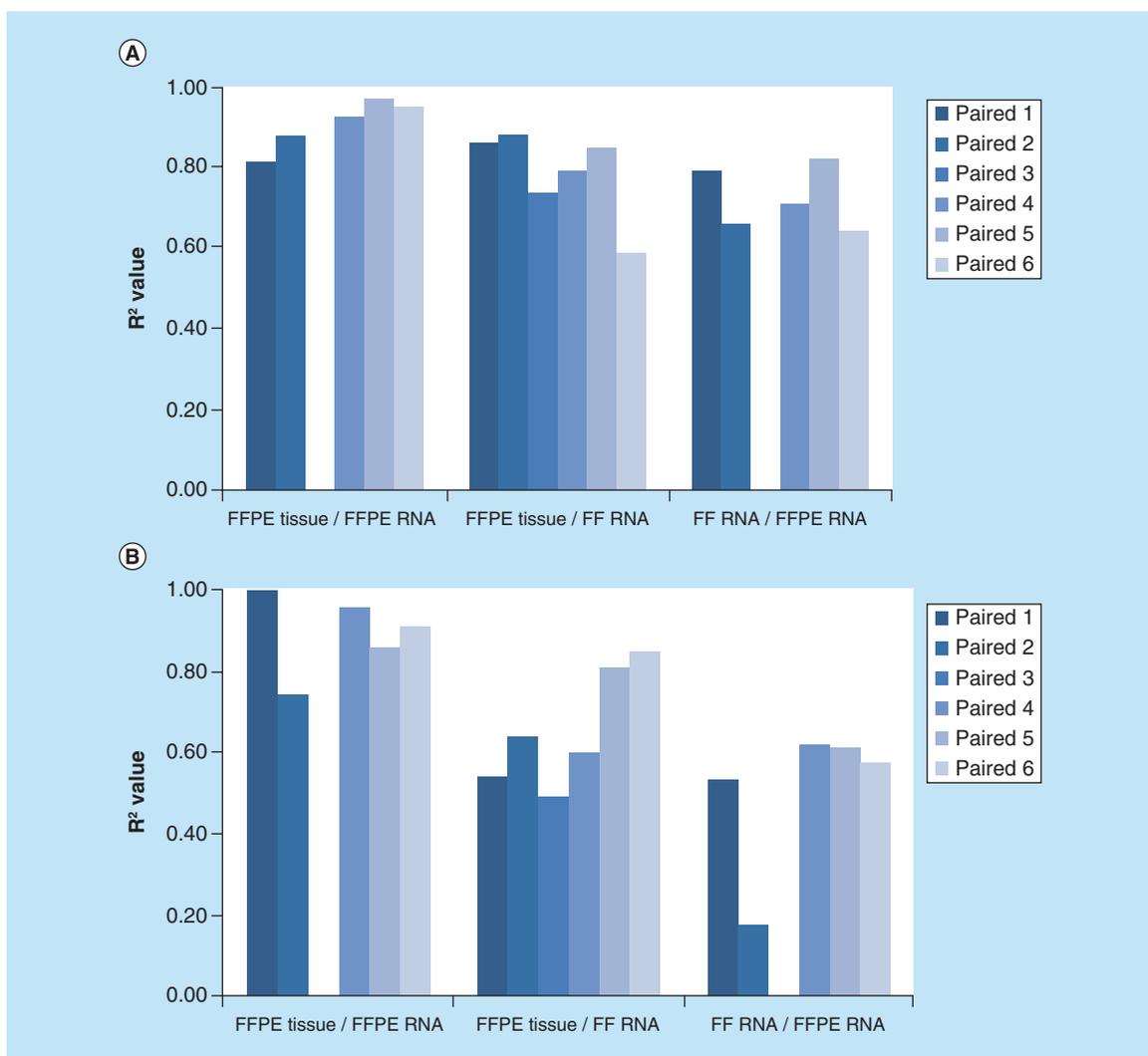
Figure 4. Correlation of normalized gene expression among matched fresh frozen RNAs, formalin-fixed, paraffin-embedded lysates and formalin-fixed, paraffin-embedded RNAs from HTG quantitative nuclease protection assay-derived or QuantiGene-derived data. Notch pathway and HK gene expressions were analyzed in six matched FF RNAs, FFPE lysates, and FFPE RNAs (FFPE RNA from pair 1–6, with pair 6 being most the fragmented) **(A)** by HTG qNPA and **(B)** by QGM. The correlations of the normalized gene expression values for the matched FFPE tissue versus FFPE RNA, FFPE tissue versus FF RNA and FF RNA versus FFPE RNA samples were determined. The R^2 values were plotted for comparison. Note that the FFPE RNA in pair 3 did not yield results.
FF: Fresh frozen; FFPE: Formalin fixed, paraffin embedded.
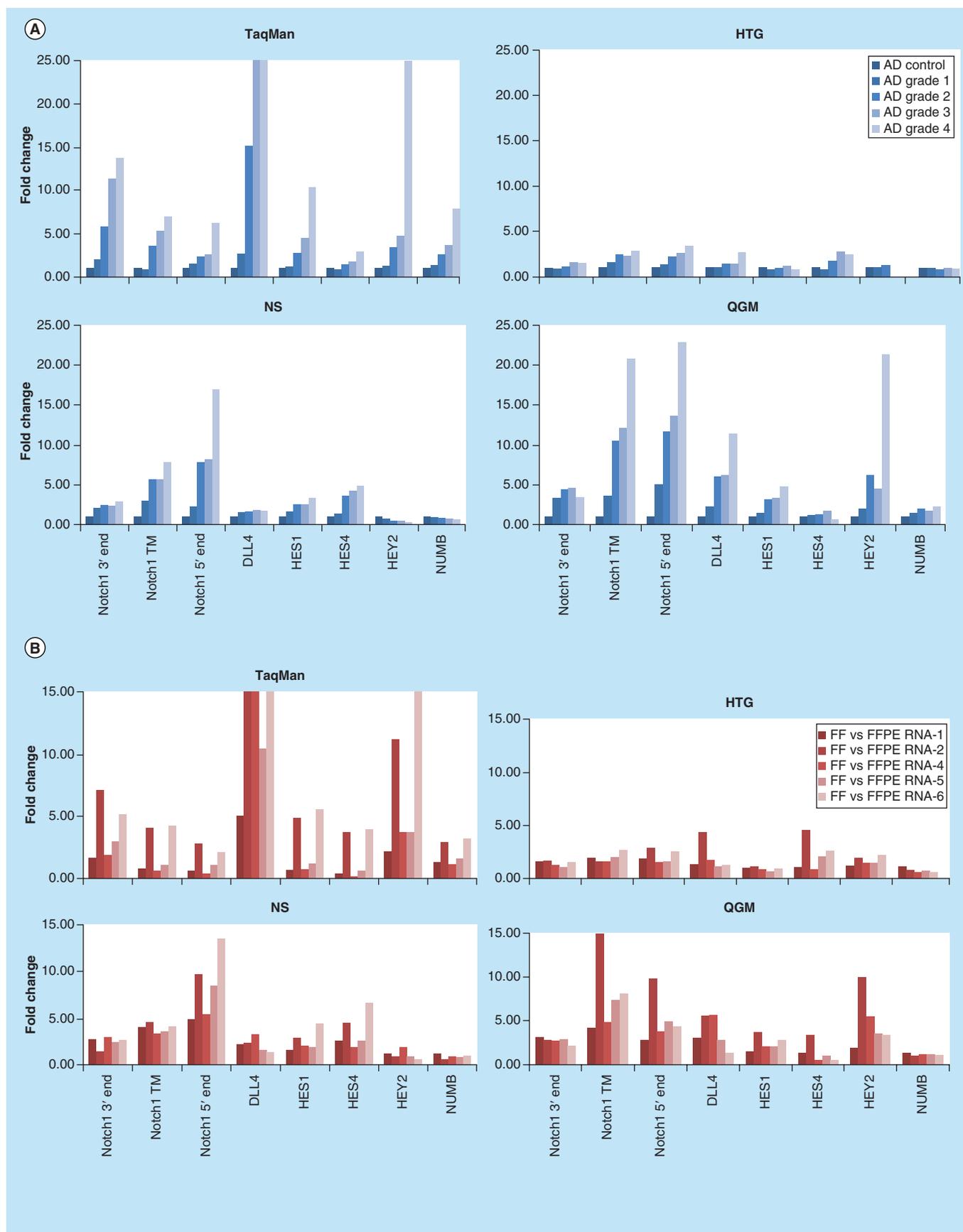
Figure 5. Consistency of the gene expression measurements in artificially degraded versus control RNAs and in formalin-fixed, paraffin-embedded versus matched fresh frozen RNAs by the four platforms (see facing page).
(A) Notch and HK gene expressions were analyzed in one control and four AD RNAs (from AD grade 1–4, with grade 4 being the most fragmented) using the four assay platforms: HTG, QGM, NS and TaqMan. Fold changes between the normalized gene expression values in the four AD RNAs versus the control RNA were calculated and plotted to compare the results for each platform. **(B)** Notch pathway and HK gene expression in five matched FFPE and FF RNAs (from FFPE RNA-1 to RNA-6, with RNA-6 being the most fragmented, no RNA-3) were analyzed using the four platforms. Fold changes between the normalized gene expression values in the FFPE versus matched FF RNAs were calculated and plotted to compare the results for each assay platform.
AD: Artificially degraded; FF: Fresh frozen; FFPE: Formalin fixed, paraffin embedded; HTG: HTG qNPA; NS: NanoString nCounter; QGM: QuantiGene.

extraction, thus increasing the detection sensitivity and decreasing the amount of FFPE tissue required for analysis. In conclusion, our study indicates that the HTG qNPA platform is uniquely suited for analyzing gene expression in clinically derived FFPE tissue samples, and has the potential to provide gene-expression data that can advance the development of clinical biomarkers for cancer.

Future perspective
FFPE tissue will continue to be a major clinical specimen for biomarker research, especially in oncology research, where the trend is toward lower sample input and higher-throughput assays that maintain reliable and sensitive detection. With the advent of next-generation sequencing (NGS) [20,21], an opportunity to further enhance qNPA arises, given that its working mechanism is compatible with the NGS workflow. The combination of these two technologies has the potential to further reduce sample input and increase detection sensitivity, the linear dynamic range and throughput. In fact, a next-generation product, called

EdgeSeq, which combines the qNPA and NGS technologies and can sequence mRNA libraries from extremely limited amounts of FFPE material, such as core-needle and fine-needle biopsies, has been developed by HTG [22,23]. This technology will add to the arsenal for research on the tumor microenvironment by enabling the analysis of small tissue regions isolated from macro or microdissection. Although these technologies offer considerable potential advantages, it is important for bioanalytical laboratories to evaluate each technology platform for its intended use.

Executive summary

Background
- Formalin-fixed, paraffin-embedded (FFPE) tissue is a type of clinical sample with the potential to provide valuable gene expression data for the development of cancer biomarkers.
- However, FFPE RNA is extensively fragmented and presents a significant challenge for reliably detecting gene expression using traditional qPCR.

Experimental
- Three novel methodologies along with the traditional qPCR method were evaluated for their ability to detect Notch pathway gene expression in six fresh frozen colorectal cancer RNA samples and matching FFPE tissue RNA samples.
- The ability of each platform to consistently detect gene expression across the RNA samples was analyzed by linear regression and R^2 correlation coefficients.

Results & discussion
- Quantitative nuclease protection assay (qNPA), with a small hybridization footprint, detected gene expression in high-quality RNAs as sensitively as qPCR, and consistently detected mRNAs in the highly fragmented FFPE tissue RNAs.
- In addition, qNPA, which does not require RNA extraction, detected gene expression in FFPE tissue lysates more accurately than in FFPE RNA samples.

Conclusion
- qNPA represents an improved methodology for detecting gene expression in FFPE tissue. It has the potential to improve detection sensitivity and reduce sample input amounts, advantages that are critical for the development of novel cancer biomarkers.

cial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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